AD_____

GRANT NUMBER DAMD17-97-1-7334

TITLE: Third International Meeting on Esterases Reacting with Organophosphorus Compounds

PRINCIPAL INVESTIGATOR: Dr. Elsa Reiner

CONTRACTING ORGANIZATION: Institute for Medical Research and Occupational Health
HR - 10001 Zagreb, Croatia

REPORT DATE: ~~November~~ 1998

TYPE OF REPORT: Final Proceedings

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	~~November~~ 1998	Final Proceedings (1 Aug 97 - 30 Sep 98)

4. TITLE AND SUBTITLE

Third International Meeting on Esterases Reacting with Organophosphorus Compounds

5. FUNDING NUMBERS

DAMD17-97-1-7334

6. AUTHOR(S)

Dr. Elsa Reiner

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Institute for Medical Research and Occupational Health
HR - 10001 Zagreb, Croatia

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS

15. NUMBER OF PAGES
47

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Third International Meeting
on
ESTERASES REACTING WITH
ORGANOPHOSPHORUS COMPOUNDS

15 - 18 April 1998
Dubrovnik – Croatia

PROGRAMME

and

ABSTRACTS

The *Third International Meeting on Esterases Reacting with Organophosphorus Compounds* is organized by the Institute for Medical Research and Occupational Health, Zagreb, Croatia. The Meeting is held under the auspices of the Ministry of Science and Technology of the Republic of Croatia and the Croatian Academy of Sciences and Arts.

Members of the *Scientific Committee* are B.P. Doctor (USA), C.E. Furlong (USA), M.K. Johnson (UK), M. Lotti (Italy), E. Reiner (Croatia), I. Silman (Israel), V. Simeon-Rudolf (Croatia) and P. Taylor (USA). Members of the *Organizing Committee* are S. Milković-Kraus, E. Reiner, V. Simeon-Rudolf, M. Škrinjarić-Špoljar and Ž. Vasilić, and the junior members S. Herceg, Z. Kovarik and A. Lucić (all from the Institute).

The Meeting has received financial support from the Ministry of Science and Technology of the Republic of Croatia, Ministry of Defence of the Republic of Croatia, US Army Medical Research Acquisition Activity (USA), Association of the European Toxicologists and Societies of Toxicology (EUROTOX; The Netherlands) and NOVARTIS Crop Protection AG (Switzerland). The Meeting also received support from sponsors listed at the end of the book.

The previous two meetings on the same subject were also organized by the Institute for Medical Research and Occupational Health. The first meeting was held in the Inter-University Centre (IUC) Dubrovnik in April 1988. Dubrovnik was also planned to host the second meeting in spring 1991. However, due to the war against Croatia the meeting had to be postponed. Dubrovnik was heavily damaged during the war and the IUC was almost completely destroyed. Our colleagues from Padua University generously offered their help and the second meeting took place in Salsomaggiore (Italy) in April 1992. The third meeting is again in Dubrovnik and the venue is the rebuilt Inter-University Centre.

Elsa Reiner

GENERAL PROGRAMME

Venue: Inter-University Centre (IUC)
Don Frana Bulića 4
Phone: ++(385-20) 413626 or 413627
Fax: ++(385-20) 413628

Tuesday, 14 April

Noon to 7.00 pm	Registration and setting posters on display
7 pm	Welcome reception at hotel "Splendid", Masarykov put 18, Phone: ++(385-20) 414173, Fax: ++(385-20) 416487

Wednesday, 15 April

8.00 am - 10.00 am	Registration and setting posters on display
10.00 am - 11.00 am	Opening of the Meeting
11.00 am - 1.00 pm	Platform presentations L01 - L04
4.00 pm - 6.30 pm	Platform presentations L05 - L09

Thursday, 16 April

9.00 am - 1.00 pm	Platform presentations L10 - L14 and P01 - P12
4.00 pm - 6.30 pm	Platform presentations L15 - L18 and P13 - P24

Friday, 17 April

9.00 am - 1.00 pm	Platform presentations L19 - L23 and P25 - P36
4.00 pm - 6.30 pm	Platform presentations L24 - L27 and P37 - P48

Saturday, 18 April

9.00 am - 2.00 pm	Platform presentations L28 - L34 and P49 - P73 Closing of the Meeting
7 pm	Farewell dinner at hotel "Splendid"

WEDNESDAY morning

L-01 P. Taylor, Z. Radić, N. Hosea, L. Wong, P. Marchot, Y. Bourne, H. Berman
Uncovering Inhibition and Reactivation Mechanisms of the Cholinesterases by Selective Ligands and Analysis of Structure

L-02 T.L. Rosenberry, T. Szegletes and W.D. Mallender
A New Mechanistic Interpretation of Acetylcholinesterase Inhibition by Peripheral Site Ligands and Substrate Inhibition

L-03 P. Masson, P-L. Fortier, C. Albaret, C. Clery, P. Guerra and O. Lockridge
Structural Changes in the Active Site Gorge of Phosphylated Butyrylcholinesterase (BuChE) Accompanying the Aging Process

L-04 J. Massoulié
Quaternary Structure and Localization of Acetylcholinesterase

WEDNESDAY afternoon

L-05 I. Silman, C.B. Millard, G. Kryger, A. Ordentlich, M. Harel, H.A. Greenblatt, Y. Segall, D. Barak, A. Shafferman and J.L. Sussman
Structural Studies on *T. californica* Acetylcholinesterase and its 'Aged' Phosphorylated and Phosphonylated Conjugates

L-06 A. Shafferman, A. Ordentlich, D. Barak, Y. Segall, N. Ariel, R. Barak and B. Velan
The Catalytic Contribution of Specific HuAChE Residues to the Aging of Phosphonyl-Enzyme Conjugates

L-07 D.M. Quinn, S. Malany, M. Verweyst, R. Medhekar, Z. Radić, P. Taylor, A. Shafferman, B. Velan and C. Kronman
Theoretical and Experimental Investigations of Electrostatic Effects on Acetylcholinesterase Catalysis and Inhibition

L-08 Z. Radić and P. Taylor
The Influence of Peripheral Site Ligands on the Reaction of Symmetric and Chiral Organophosphates with Wild-Type and Mutant Acetylcholinesterases

L-09 V. Simeon-Rudolf, Z. Kovarik, Z. Radić and E. Reiner
Reversible Inhibition of Acetylcholinesterase and Butyrylcholinesterase by a Coumarin Derivative and by 4,4′-Bipyridine

THURSDAY morning

L-10 O. Lockridge
Acetylcholinesterase Knockout Mouse, Butyrylcholinesterase Tetramers, and Paraoxonase Active Site

L-11 C.A. Broomfield, O. Lockridge and C.B. Millard
Design and Construction of Butyrylcholinesterase Mutants that have Organophosphorus Acid Anhydride Hydrolase Activity

L-12 F.M. Raushel
Stereochemical Contraints on the Hydrolysis of Organophosphate Nerve Agents by Phosphotriesterase

L-13 B. diSioudi, C.L. Miller, K. Lai, L. Scapozza, J.K. Grimsley and J.R. Wild
Rational Design of Organophosphate Hydrolase for Altered Substrate Specificities

L-14 C.E. Furlong, R.J. Richter, W.F. Li and L.G. Costa
Paraoxonase (Ponl) Polymorphism – What's Important, Genotype, Phenotype or both?

P-01 A. Saxena, A.M.G. Redman, X. Jiang, O. Lockridge and B.P. Doctor
Differences in Active-Site Gorge Dimensions of Cholinesterases Revealed by Binding of Inhibitors to Human Butyrylcholinesterase

P-02 A. Ordentlich, D. Barak, C. Kronman, N. Ariel, B. Velan and A. Shafferman
Does Electrostatic Attraction or Steering by Charged Residues Within the Gorge Contribute to the Reactivity of AChE?

P-03 A. Ordentlich, R. Barak, D. Barak, M. Fischer, H. P. Benschop, L.P.A. De Jong, Y. Segall, B. Velan and A. Shafferman
ESMS as a Unique Tool for the Molecular Monitoring of Reactions Between HuAChE and Various OP-Agents

P-04 D. Barak, A. Ordentlich, Y. Segall, B. Velan, H.P. Benschop, L.P.A. De Jong. and A. Shafferman
The Aromatic Moiety at Position-86 of HuAChE Accelerate the Aging of Phosphonyl-AChE Conjugates through Cation-π Interactions

P-05 C.Varkey-Altamirano and O. Lockridge
Association of Tetramers of Human Butyrylcholinesterase is Mediated by Conserved Aromatic Residues of the Carboxy Terminus

P-06 Z. Radić
Molecular Modeling of Transition States in Acetylcholinesterase and Butyrylcholinesterase Inhibition by Carbamates

P-07 Z. Radić, M. Šentjurc, S. Pečar, J. Stojan and Z. Grubič
EPR Labeling of Acetylcholinesterase-fasciculin2 Complex: Molecular Models and Experiment

P-08 C. Cléry, J. Clément, P. Guerra and P. Masson
Effect of Hydrostatic and Osmotic Pressures on the Rate of Aging of DFP-Phosphorylated Wild-Type and Mutants (E197D and D70G) of Butyrylcholinesterase

P-09 H. Shao, Y. Haung, D. Wang, H. Zhang and M. Sun
Grafting of Genetically Modified Human Fetal Fibroblast to Produce Human Butyrylcholinesterase in Mice

P-10 T. Szegletes, W.D. Mallender and T.L. Rosenberry
Nonequilibrium Analysis of Acetylcholinesterase Inhibition by Propidium and Gallamine

P-11 C. Luo, A. Saxena, Z. Radić, Y. Ashani, P Taylor and B.P. Doctor
Role of Edrophonium in Prevention of the Reinhibition of Acetylcholinesterase by Phosphorylated Oxime

P-12 C. Luo, Y. Ashani and B.P. Doctor
Acceleration of Oxime-Induced Reactivation of MEPQ-Inhibited Acetylcholinesterase by Mono- and Bis-Quaternary Ligands

THURSDAY afternoon

L-15 B. N. La Du, M. Aviram, S. Billecke, M. Navab, S.L. Primo-Parmo, R.C. Sorenson and T.J. Standiford
On the Physiological Role(s) of the Paraoxonases

L-16 L.G. Costa, W.F. Li, R.J. Richter, A. Lusis, D.M. Shih and C.E. Furlong
Paraoxonase (Pon1) and Sensitivity to Organophosphate Toxicity: Animal Studies

L-17 F.C.G. Hoskin, J.E. Walker and C.M. Mello
Organophosphorus Acid Anhydrolase (OPAA) in Slime Mold, Duckweed and Mung Bean: a Continuing Search for a Physiological Role and a Natural Substrate

L-18 D.M. Maxwell, A. Saxena, A.J. Russell and B.P. Doctor
Improvements in Scavenger Protection Against Organophosphorus Agents by Modification of Cholinesterases

P-13 Z. Kovarik, M. Škrinjarić-Špoljar, B. Grgas, Z. Radić and V. Simeon-Rudolf
Amino Acids Involved in the Inhibition of Acetylcholinesterase and Butyrylcholinesterase by Ro 02-0683 and Bambuterol

P-14 V. Simeon-Rudolf, Z. Kovarik, A. Buntić, M. Škrinjarić-Špoljar and R.T. Evans
Kinetics of Inhibition of Human Serum Butyrylcholinesterase (EC 3.1.1.8) Heterozygous Phenotypes by the Dimethylcarmabate Ro 02-0683

P-15 V. Simeon-Rudolf, E. Reiner, R.T. Evans, P. George and H. Potter
Catalytic Parameters for the Hydrolysis of Butyrylthiocholine by Human Serum Butyrylcholinesterase Phenotypes

P-16 D.Josse, W.H. Xie, P. Masson, L.M. Schopfer and O. Lockridge
Tryptophan Residue(s) as Major Component(s) of the Human Paraoxonase Active Site

P-17 D. Josse, W.H. Xie, P. Masson and O. Lockridge
Human Serum Paraoxonase: Identification of Essential Amino Acid Residues by Group-Specific Labelling and Site Directed Mutagenesis

P-18 W.H. Xie, P.J. Wilder, J. Stribley, A. Chatonnet, A. Rizzino, P. Taylor, S. Hinrichs and O. Lockridge
Acetylcholiesterase Knockout Mouse

P-19 J.A. Doorn, R.C. Sorenson, S.S. Billecke, C. Hsu and B.N. La Du
Evidence That Several Conserved Histidine Residues are Required for Hydrolytic Activity of Human Paraoxonase/Arylesterase

P-20 M.A. Sogorb, A. Monroy, J.L.Vicedo, J. Barril and E. Vilanova
Serum Albumin Can Hydrolyse Organophosphorus Compounds by an Intermediate Phosphorylated Mechanism. Where is the Borderline Dividing A- and B-Esterases?

P-21 R.C. Sorenson, M. Aviram, C.L. Bisgaier, S. Billecke, C. Hsu and B.N. La Du
Properties of the Retained N-Terminal Hydrophobic Leader Sequence in Human Serum Paraoxonase/Arylesterase

P-22 A.F. Hernández, J.J. López, L. Rodrigo, F. Gil and A. Pla
Immunolocalization of Paraoxonase in Rat Tissues

P-23 L. Rodrigo, F. Gil, A.F. Hernández and A. Pla
Identification of Two Rat Liver Proteins with Paraoxonase Activity

P-24 L. Rodrigo, F. Gil, A.F. Hernández and A. Pla
Further Evidence for the Identity of Paraoxonase and Arylesterase Activities in Rat Liver

FRIDAY morning

L-19 M. Lotti
Promotion of Axonopathy by Certain Esterase Inhibitors

L-20 A. Moretto
The Search for the Target of Promotion of Axonopathies

L-21 E. Vilanova, M.A. Escudero and J. Barill
NTE Soluble Isoforms: New Perspectives for Targets of Neuropathy Inducers and Promoters

L-22 P. Richards, C. Walker, D. Ray and M. Johnson
Novel Protein Targets for Organophosphorus Compounds

L-23 P. Glynn, D.J. Read, M.J. Lush, J.M. Atkins and Y. Li
Molecular Cloning of Neurophaty Target Esterase (NTE)

P-25 J. Barril, J.Estévez, M.A. Escudero, M.V. Céspedes, N. Ňíguez,.A. Sogorb, M.A. Monroy and E. Vilanova
Peripheral Nerve Soluble Esterases are Spontaneously Reactivated after Inhibition by Some OP Compounds: Implications for a New Definition of NTE

P-26 M.A. Escudero, J. Barril, M-V. Céspedes and E. Vilanova
Chicken Liver Phenyl Valerate Esterases Discriminated by Paraoxon and Mipafox Inhibition

P-27 L.V. Sigolaeva, A.V. Eremenko, G.F. Makhaeva, V.V. Malygin and I.N. Kurochkin
A New Way for Determination of Neuropathy Target Esterase Activity

P-28 G.F. Makhaeva and V. Malygin
A Stable Preparation of Hen Brain Neuropathy Target Esterase for Rapid Biochemical Assessment of Neurotoxic Potential of Organophosphates (OP)

P-29 G.F. Makhaeva, S. Fomicheva, V. Malygin, A. Kharitonov and A. Yarkevich
Aryl Diarylphosphinates as Inhibitors of Hen Brain Neuropathy Target Esterase

P-30 V. Malygin and G. Makhaeva
Protective Role of Blood and Liver Esterases in Toxic Action of O,O-Dialkyl-S-Carboethoxybromomethylthiol Phosphates

P-31 J. Vodiškar, V. Zgonc, S. Ribarič and Z. Grubič
Glucocorticoids and Electromechanical Activity Independently Regulate Acetycholinesterase in the Mammalian Skeletal Muscle

P-32 U. Weber, M. Brank and Z. Grubič
Glucocorticoides Differentially Control Synthesis of Acetylcholinesterase and Butyrylcholinesterase in Rat Liver and Brain

P-33 K. Zajc-Kreft, S. Kreft and Z. Grubič
Degradation Rates of AChE mRNA in the Normal and Denervated Rat Fast Skeletal Muscle, Studied *in vitro*

P-34 T. Milanič, A. Kunstelj, T. Marš and Z. Grubič
Myonuclear Density and Distribution in the Normal and Denervated Rat Fast Skeletal Muscle

P-35 K. Miš, K. Zajc-Kreft and Z. Grubič
Localization of Cells Expressing AChE mRNA in Rat Brain Using Nonradioactive *in situ* Hybridization Combined with Fluorescent Nuclear Labeling

P-36 K. Sepčić, N. Poklar, G. Vesnaver, T. Turk and P. Maček
Chemical and Physical Studies of Acetylcholinesterase Inhibition by Its Naturally Occurring Inhibitor, Poly-APS

FRIDAY afternoon

L-24 G. Amitai, D. Moorad, R. Adani and B.P. Doctor
Chlorpyrifos-Oxon is a Rapid Inhibitor of BChE

L-25 V. Marcel, D. Fournier and J. Stojan
Inhibition of *Drosophila* Acetylcholinesterase by 7-(Methylethoxyphosphinyloxy) 1-Methyl-Quinolinium Iodide (MEPQ)

L-26 M.I. Mackness
Antiatherogenic Properties of Human Serum Paraoxonase (PON)

L-27 H. Leader, N. Rothschild, C. Dosoretz and Y. Ashani
Organophosphorus Hydrolases as Antidotes Against Organophosphates Toxicity: A Phosphorylated Oxime is Substrate of Bacterial Phosphotriesterase

P-37 R. Zech, R.M. Severin, J.M. Chemnitius and K. Nebendahl
Paraoxonase Polymorphism in Rabbits

P-38 B. Mackness, M.I. Mackness, S. Arrol, W. Turkie and P.N. Durrington
Molecular Polymorphisms of Human Serum Paraoxonase (PON) Affect Activity

P-39 B. Mackness, R. Hunt, P.N. Durrington and M.I. Mackness
Immunolocalisation of Paraoxonase with Clusterin and Apolipoprotein A1 in the Human Artery Wall

P-40 E. Reiner, D. Svedružić, V. Simeon-Rudolf, V. Lipovac, M. Gavella and V. Mrzljak
Paraoxonase and Arylesterase Activities in the Serum of Two Hyperlipoproteinaemic Patients after Repeated Extracorporal Lipid Precipitation

P-41 U. Helfrich, A. Schmitt, M. Toomes, G. Petroianu and R. Ruefer
Paraoxon (POX) Inhibits Phospholipase A_2 (PLA_2)

P-42 U. Helfrich, G. Petroianu, W. Bergler, J. Fisher and R. Ruefer
Acetylcholine Inhibits PLA_2 Activity

P-43 G. Petroianu, U. Helfrich, J. Fisher, W. Bergler and R. Ruefer
Phospholipase A_2 (PLA_2) Activity in Mini Pigs After Acute Paraoxon (POX) Intoxication

P-44 G. Petroianu, U. Helfrich, M. Toomes and R. Ruefer
Paraoxon Does Not Inhibit α-Lactamase

P-45 M. Toomes, G. Petroianu, W Bergler, A. Petroianu and R. Ruefer
Control of Blood Pressure (BP), Heart Rate (HR) and Hematocrit (Hct) during High Dose *iv*-Paraoxon (POX) Exposure in Mini Pigs

P-46 M. Toomes, G. Petroianu, W. Maleck, W. Bergler and R. Ruefer
High Dose Intravenous Paraoxon (POX) Exposure: Coagulation Studies in Mini Pigs

P-47 P. Santhoshkumar and T. Shivanandappa
Differantial Sequestration of Two Organophosphorus Homologs by the Rat Liver Homogenate

P-48 S.S. Billecke, S.L. Primo-Parmo, C.S. Dunlop, J.A. Doorn, B.N. La Du and C.A. Broomfield
Characterization of a Soluble Mouse Liver Enzyme Capable of Hydrolyzing DFP (Diisopropyl Phosphorofluoridate)

SATURDAY

L-28 J. Sketelj and N. Črne-Finderle
AChE in the Neuromuscular Junctions

L-29 K. Zajc-Kreft, T. Marš, M. Brank, A.F. Miranda and Z. Grubič
Control Points of Acetylcholinesterase Synthesis in the Mammalian Skeletal Muscle

L-30 D. Kaufer, A. Friedman, S. Seidman and H. Soreq
Organophosphate Inhibition of Acetylcholinesterase Induces a Molecular/Physiological Cascade of Feedback Events Leading toward Long-Term Impairments in Cholinergic Functions

L-31 T. Satoh, S. Suzuki, N. Kawai, T. Nakamura, M. Hosokawa
Toxicological Significance in the Cleavage of Esterase-β-glucuronidase Complex in Liver Microsomes by Organophosphorus Compounds

L-32 E. Heymann
Estimation of Inhibitory Organophosphates with Purified Pig Liver Carboxylesterase

L-33 A. Chatonnet, T. Hotelier and X. Cousin
Kinetic Parameters and Structure-Function Data Retrieval through ESTHER, the Database on Cholinesterases and α/β Hydrolases

L-34 S. Estrada-Mondaca, D. Fournier, A. Klaebe, F. Magné, V. Marcel, P. Masson, J. Stojan and F. Villatte
Metabolisation of Substrate by Invertebrate Cholinesterase

P-49 A.F. Hernández, M.C. Gonzalvo, F. Gil, L. Rodrigo, E. Villanueva and A. Pla
Distribution Profiles of Paraoxonase and Cholinesterase Phenotypes in a Spanish Population

P-50 Z. Šiftar, Z. Flegar-Meštrić and B. Šurina
Biological Variations of Serum Cholinesterase Activity in Population of Zagreb, Croatia

P-51 F.M. Williams and E. Mutch
Organophosphate Toxicity to Man – Inter Individual Differences in Susceptibility

P-52 I. Primožič, R. Odžak and S. Tomić
3-Hydroxyquinuclidinum Derivatives: Synthesis and Structures

P-53 E. Reiner, M. Škrinjarić-Špoljar, S. Dunaj, A. Buntić and V. Simeon-Rudolf
3-Hydroxyquinuclidinum Derivatives: Reversible and Acylating Inhibitors of Acetycholinesterase

P-54 J. Lange, S. Rump, T. Gidyńska, E. Gałecka, M. Kowalczyk and B. Serafin
Chemical Structure - AChE Protective Activity Relationship Among a Series of Bis-Pyridinium Mono- and Di-Oximes

P-55 V. Tonkopii
Peculiarities of Interaction of Reversible Inhibitors with Acetylcholinesterase and Effectiveness of Prophylaxis

P-56 M. Ghazi-Khansari, A. Amoshahie, M. Faizie and M. Rahimie
Esterases Activity in Organophosphorus Poison Patient

P-57 J. Matoušek
Catalytic Models with Cholinesterase Activity

P-58 J. Matoušek
Methods for the Determination of Organophosphorus Compounds Based on Cholinesterase Inhibition Using Non-Biogenic Chromogenic and Fluorogenic Substrates

P-59 J. Matoušek
New Ways of the Detection of Organophosphorus Compounds Based on Cholinesterase Inhibition with Thiocholine Esters as Substrates

P-60 Ž. Vasilić, B. Štengl and V. Drevenkar
Dimethylphosphorus Metabolites in Serum and/or Urine of Persons Poisoned by Malathion or Thiometon

P-61 R.K. Gordon, S.R. Feaster, D.E. Lenz, A.J. Russell and B.P. Doctor
Organophosphate Skin Decontamination Using Immobilized Enzymes

P-62 W.D. Dettbarn, Z.P. Yang and D. Milatović
The Role of Carboxylesterase in Development of Resistance to Paraoxon

P-63 P.Y. Renard, P. Vayron, F. Taran, C. Creminon, Y. Frobert, J. Grassi and C. Mioskowski
Studies on the Degradation of Acetylcholinesterase Organophosphorus Inhibitors Using Catalytic Antibodies

P-64 T. Cheng, J.J. DeFrank and V.K. Rastogi
Alteromonas Prolidase for Organophosphorus G-Agent Decontamination

P-65 F.C.G. Hoskin, J.E. Walker and R.Stote
Degradation of Nerve Gases by CLECs and Cells: Kinetic of Heterogenous Systems

P-66 J.M. Chemnitius, R. Sadowski, H. Winkel and R. Zech
Organophosphate Inhibition of Human Heart Muscle Cholinesterase Isoenzymes

P-67 A. Monroy, M.A. Sogorb, N. Díaz-Alejo, J.L. Vicedo, J. Barill and E. Vilanova
Dichlorophenyl Phosphoramidates as Substrates for Phosphotriesterases in Avian and Other Species: Variability in Level, Calcium Dependence and Stereoespecificity

P-68 M. Srivatsan
Effects of Organophosphates on Cholinesterase Activity and Neurite Regeneration in *Aplysia*

P-69 V. Petrović-Peroković, J. Tomašić, Z. Mihalić and S. Tomić
Purification and Kinetics of Guinea-Pig Esterases Hydrolyzing Esterified Monosaccharides

P-70 L. Senčič and B. Štih
Inhibition of the Acid Phosphatase in the Seeds of *Pulsatilla grandis* with Zeolite A

P-71 M. Krsnik-Rasol, H. Čipčić
Isoesterases Related to Cell Differentiation in Plant Tissue Culture

P-72 A. Küthe, H. Eckel, H-J. Mägert, C.G. Stief, S. Ückert, W-G. Forssmann and U. Jonas
Molecular Characterization of Phosphodiesterases in Human Corpus Cavernosum

P-73 F. Villatte, V. Marcel, S. Estrada-Mondaca and D. Fournier
Engineering Sensitive Acetylcholinesterase for Detection of Organophosphate and Carbamate Insecticides

L 01

UNCOVERING INHIBITION AND REACTIVATION MECHANISMS OF THE CHOLINESTERASES BY SELECTIVE LIGANDS AND ANALYSIS OF STRUCTURE.

Palmer Taylor[1], Zoran Radić[1], Natilie Hosea[1], Lilly Wong[1], Pascale Marchot[1,2], Yves Bourne[1,2] and Harvey Berman[3]. [1]Dept. of Pharmacology, University of California, San Diego, [2]University of Marseille, France, [3]Dept. of Biochemical Pharmacology, SUNY, Buffalo.

The availability of crystal structures of *Torpedo* and mouse acetylcholinesterase (AChE) enables one to analyze ligand selectivity in relation to a detailed structural template. To this end, we have examined the kinetics of inhibition of AChE by series of R- and S-alkyl methylphosphonylthioates. The alkyl group modifications include isopropyl, 2,2-dimethyl butyl and cycloheptyl and the thioate leaving groups were thiocholine, ethanethiol, and methanethiol. The two diastereomeric conjugates that formed were examined for their reactivation rates using several oximes. The acyl pocket, the choline binding site and peripheral site in AChE were modified through site-specific mutagenesis, and thermodynamic mutant cycle analysis was used to delineate pairwise interactions between residues on the enzyme and the phosphonate. Herein, we find that acyl pocket dimensions affect enantiomeric selectivity of the phosphonates, and a carboxylate (D74) residing just below the active center gorge opening governs specificity of the phosphonates with charged and uncharged leaving groups. Oxime reactivation kinetics show reactivator specific selectivity for the two diastereomers and suggest that the attacking orientations of the oximes differ for the reactivation scheme.

The 6 kDa peptide fasciculin from snake venom binds near the active center gorge entrance of AChE to restrict, but not totally occlude, substrate entry. By using diffusion and non-diffusion limited substrates such as trimethyl ammoniotrifluoroacetophenone, it is possible to change the rate limitations and pathway effecting substrate entry into the gorge therein modifying the barriers for rate limitation of catalysis. Through an analysis of ionic strength dependence and mutations of fasciculin, the projected routes of substrate entry and contact points between the inhibitory toxin and the enzyme can be ascertained.

(Supported by USPHS GM18360 and DAMD17-951-5027)

L 02

A new mechanistic interpretation of acetylcholinesterase inhibition by peripheral site ligands and substrate inhibition

Terrone L. Rosenberry, Tivadar Szegletes and William D. Mallender

Department of Pharmacology, Mayo Foundation for Medical Education and Research, and the Department of Research, Mayo Clinic Jacksonville, Jacksonville, FL 32224, USA

Many ligands that bind to the peripheral site of acetylcholinesterase (AChE) inhibit substrate hydrolysis at the acylation site, though the interpretation of this inhibition has been unclear. Since previous explanations based upon equilibrium ligand binding cannot be justified for AChE, we introduce here an alternative nonequilibrium analysis. This analysis incorporates a steric blockade mechanism which assumes that the only effect of a small bound peripheral ligand is to slow down the rate of substrate association and dissociation at the acylation site and concomitantly reduce the product dissociation. Experimental data using propidium and gallamine as peripheral site ligands and acetylthiocholine, phenyl acetate, huperzine A and *m*-(*N,N,N*-trimethylammonio)trifluoroacetophenone as acylation site ligands agreed well with our theory. The nonequilibrium model has been extended to account for substrate inhibition based upon acetylthiocholine affinity for the peripheral site. This affinity was determined by measuring acetylthiocholine inhibition of the association rate k_{on} for the slowly equilibrating peripheral site ligand fasciculin 2. The measured K_s of about 1-2 mM was considerably lower than the K_{ss} estimate of about 20 mM determined by application of the Haldane equation to the steady-state substrate inhibition curve. A better understanding of the interaction between peripheral site ligands and acetylcholinesterase may help to understand the physiological role of the peripheral site and to design new ligands that inhibit organophosphorylation.

L 03

STRUCTURAL CHANGES IN THE ACTIVE SITE GORGE OF PHOSPHYLATED BUTYRYLCHOLINESTERASE (BuChE) ACCOMPANYING THE AGING PROCESS

P. Masson[a], P-L. Fortier[b], C. Albaret[b], C. Clery[a] P. Guerra[a] and O. Lockridge[c]
[a.] Centre de Recherches du Service de Sante des Armees, Unite d'Enzymologie, BP 87, 38702 La Tronche, France; [b.]Centre d'Etudes du Bouchet, 91710 Vert-le-Petit, France; [c.] University of Nebraska Medical Center, Eppley Inst., Omaha, NE 68198, USA.

Dealkylation of an alkoxy chain on the P atom of phosphylated ChEs leads to 'aged' enzymes which cannot be reactivated. The mechanism of the dealkylation and the molecular basis of the resistance of aged ChEs to reactivators are not completely understood. It has been shown that aged ChEs are more stable than native ones [1], but the effects of changes in the active center (at least a salt bridge between H438 and PO^-) on the overall structure have not been completely established. Wild-type (wt) BuChE and the E197D and D70G mutants were inhibited by DFP or soman under standard conditions of pH, temperature and pressure. Aging half-times of DIP-mutants were found 6-8 fold higher than that of wt ($t_{1/2}$=lh) [2]. Hydrostatic pressure increased the rate of aging, allowing to determine activation volumes ($\Delta V^{\neq}$) for the dealkylation reaction. $\Delta V^{\neq} << 0$ for DIP-wtBuChE, and $\cong$ -3.5 ml/mol for the E197D and D70G mutants, indicating that the transition state was associated with an extended conformational/hydration change in wtBuChE but not in mutants. The rate of aging decreased with osmotic pressure, suggesting hydration changes. To understand $\Delta V^{\neq}$ and behaviour differences between wt and mutants, molecular dynamics simulation under pressure was undertaken. Efforts are in progress to relate kinetic data with changes in water structure in the enzyme active site gorge. The pH dependence of the melting temperature (Tm) of native and soman-wtBuChE, as determined by DSC, indicated that stabilization energy of aged BuChE is mainly due to the salt bridge, with pK_{H438} = 8.5-9. Electrophoresis and ANS binding under high pressure up to 2.5 kbar showed that aged BuChE did not undergo pressure-induced molten globule transition compared to native enzyme. Moreover, this transition was not seen for the mutant enzymes, indicating that mutants are resistant to penetration of water into their structure. All results support the conclusion that D70 and E197 are major residues for the water/H-bond network dynamics in the active site gorge of BuChE.

[1]:P. Masson et al., J. *Mol.* Biol., (1994) 238, 466; [2]:P. Masson et al., *Biochem.* J., (1997) 327, 601. Supported by DSP/DGA grant ± 97/12 to P. M.

L 04

Quaternary structure and localization of acetylcholinesterase

Jean Massoulié

Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS URA 1857,
Ecole Normale Supérieure, 75005 Paris, France

A correct positioning of acetylcholinesterase (AChE) conditions its ability to efficiently hydrolyze acetylcholine in cholinergic synapses. In addition, AChE exists in other contexts, e.g. on blood cells, in the plasma, and in the venoms of some snakes. Evidence is presently accumulating that cholinesterases may be endowed with non-catalytic functions, e.g. in cell interactions and morphogenesis. The molecular forms of AChE correspond to soluble states and to various modes of anchoring in cell membranes or in extracellular structures. In vertebrates, these molecules are generated from a single gene: the catalytic domain may be associated with several types of C-terminal peptides, that determine subsequent post-translational events of the catalytic subunits, i.e. $AChE_S$, $AChE_H$, $AChE_T$.

$AChE_S$ generates soluble monomers, in the venom of Elapid snakes.

$AChE_H$ generates GPI-anchored dimers, in *Torpedo* muscles and on mammalian blood cells.

$AChE_T$ is the only type that exists in AChEs of all vertebrates, as well as in BChEs, and produces the major cholinesterase forms in adult brain and muscle. $AChE_T$ generates multiple structures, ranging from monomers and dimers to collagen-tailed and hydrophobic-tailed forms, in which catalytic tetramers are associated with anchoring proteins that attach them to the basal lamina or to cell membranes. In the collagen-tailed forms, $AChE_T$ subunits are associated with a specific collagen, ColQ, which is encoded by a single gene in mammals. ColQ contains a short peptidic motif, the proline-rich attachment domain (PRAD), that triggers the formation of $AChE_T$ tetramers, from monomers and dimers. The critical feature of this motif is the presence of a string of prolines, and in fact synthetic polyproline shows a similar capacity to organize $AChE_T$ tetramers. Although the COLQ gene produces multiple transcripts, it does not generate the hydrophobic tail, P, which anchors AChE in mammalian brain membranes.

An understanding of these quaternary associations is essential since the coordinated expression of $AChE_T$ subunits and anchoring proteins determines the pattern of molecular forms and therefore the localization and functionality of the enzyme.

L 05

Structural Studies on *T. californica* Acetylcholinesterase and its 'Aged' Phosphorylated and Phosphonylated Conjugates. I.

Silman[1], C.B. Millard[1,2], G. Kryger[3], A. Ordentlich[4], M. Harel[3], H.A. Greenblatt[3], Y. Segall[4], D. Barak[4], A. Shafferman[4] & J.L. Sussman[3,5].
Depts. of Neurobiology[1] and Structural Biology[3], Weizmann Institute of Science, Rehovoth 76100, Israel; [2]USAMRICD, Aberdeen Proving Ground, MD 21010, USA; [4]IIBR, Ness Ziona 70450, Israel; [5]Biology Dept., Brookhaven National Laboratory, Upton, NY 11973, USA.

The 3D structure of *T. californica* AChE (*Tc*AChE) showed that its catalytic triad is at the bottom of a narrow cavity, *ca.* 20Å deep, named the 'active-site gorge', lined by the rings of 14 highly conserved aromatic residues which play important structural and functional roles. Organophosphates (OPs) react rapidly with the active-site serine of AChE, generating a covalent conjugate which may then undergo spontaneous dealkylation, producing an 'aged' conjugate refractory to oxime reactivation. X-ray data were collected from trigonal crystals of 'aged' OP-*Tc*AChE conjugates obtained by reaction with DFP, sarin or soman. The structures refined to 2.2Å (DFP), 2.5Å (sarin) and 2.2Å (soman) resolution. The stability of the 'aged' conjugates derives from interaction of the two resonance oxygen atoms attached to the P atom with catalytic subsites. One oxygen is stabilized by H-bonding to the backbone amides of G118, G119 and A201, in the oxyanion hole; the other by a salt-bridge with the H440 imidazolium. The absolute configuration around the P atom in both phosphonyl conjugates is identical and consistent with inversion after an in-line displacement reaction with the rapidly binding P_s-stereoisomers. Whereas the backbone structures of the phosphonyl conjugates are essentially identical to that of native *Tc*AChE, in the aged DFP conjugate the residual isopropyl group distorts the acyl pocket, producing a conformational change in the loop containing residues F288 and F290 which may explain the reduced affinity of AChE, relative to butyrylcholinesterase, for DFP.

L 06

The Catalytic Contribution of Specific HuAChE Residues to the Aging of Phosphonyl-Enzyme Conjugates

Avigdor Shafferman[1], Arie Ordentlich[1], Dov Barak[1], Yoffi Segall[2], Naomi Ariel[1], Ruth Barak[3] and Baruch Velan[1]
Departments of [1]Biochemistry & Molecular Biology, [2]Organic Chemistry and [3]Analytical Chemistry, Israel Institute for Biological Research, Ness-Ziona, 70450, Israel.

The molecular species resulting from dealkylation of methylphosphonyl-HuAChE conjugates were recently identified by electrospray mass spectrometry (ESMS). Mutagenesis studies demonstrated that "aging" process is facilitated by the functional architecture of the AChE active center with specific elements participating in the different stages of the reaction mechanism. The catalytic triad element His447 is probably involved in proton transfer to the methylphosphonyl moiety resulting in a transient oxonium species. Stabilization of this oxonium - imidazolium pair may be achieved through participation of residues E202 and F338. Replacement of each of these amino acids by alanine resulted in an about 100-fold decrease of the rate of aging. It is suggested that the imidazolium moiety is maintained proximal to the phosphonyl alkoxy oxygen by electrostatic interaction with E202 and cation-π interaction with F338. The importance of the position of E202 carboxylate relative to H447 is suggested also by: a. the marked decrease in the rate of aging due to mutation E202D where the negative charge was preserved; b. the decrease in the rate of aging due to substitution of residues F450 and Y133 which are believed to interact with E202 through the hydrogen-bond network. Following the formation of the oxonium ion the dealkylation process is generally assumed to proceed via charge separation and formation of carbocationic species. Mutagenesis data suggest that the main stabilization for this step is provided by interaction with the aromatic system of residue W86. Substitution of W86 by aliphatic residue (W86A) resulted in an over 3-orders of magnitude decrease in the rate of aging. The involvement of residue Trp86 in interaction with charged species during the aging process is also indicated by the indirect involvement of the peripheral anionic site residue Asp74. Comparison of the rates of aging for methylphosphonyl-adducts of the wild type, W86F and W86A HuAChE's, with varying branching of the alkoxy substituent, further support the notion that the aromatic moiety of the residue at position-86 accommodates the evolving carbocation through cation-π interactions.

L 07

Theoretical and Experimental Investigations of Electrostatic Effects on Acetylcholinesterase Catalysis and Inhibition. Daniel M. Quinn, Siobhan Malany, Michelle Verweyst and Rohit Medhekar, Department of Chemistry, The University of Iowa, Iowa City, IA 52242 USA; Zoran Radić and Palmer Taylor, Department of Pharmacology, University of California, San Diego, CA 92093 USA; Avigdor Shafferman, Baruch Velan and Chanoch Kronman, Israel Institute for Biological Research, Ness-Ziona 70450, Israel

The role of electrostatics in the function of acetylcholinesterase (AChE) has been investigated by both theoretical and experimental approaches. Catalytic rate constants for acetylthiocholine turnover and for binding rate constants and equilibrium dissociation constants for the transition state analog inhibitor *m-N,N,N*-trimethylammoniotrifluoroacetophenone (TMTFA) have been measured for various wild-type and mutant AChEs. The free energy profiles for cationic ligand release from the active sites of wild-type and mutant AChEs have been calculated via a model that utilizes the x-ray structure of *Torpedo californica* AChE, a spherical ligand, and energy terms that account for electrostatic and Van der Waals interactions. These calculations indicate that EA and EI complexes are not bound with respect to electrostatic interactions, which obviates the need for a "back door" for cationic ligand release. Moreover, the computed energy barriers for ligand release give linear free-energy correlations with $\log(k_{cat}/K_m)$ for substrate turnover and $\log(K_i)$ for inhibition, which supports the general correctness of the computational model. The refractoriness of aged phosphonylated AChE to reactivation was also investigated by *ab initio* quantum mechanical methods. These calculations show that the aged complex is stabilized by two factors: a) the traditional electrostatic mechanism, whereby interaction with the uninegative phosphonyl moiety stabilizes His443(*440*) in its histidinium ion form; b) disruption of a low-barrier hydrogen bond (LBHB) between His443(*440*) and Glu334(*327*), which disarms the catalytic power of the enzyme.

L 08

THE INFLUENCE OF PERIPHERAL SITE LIGANDS ON THE REACTION OF SYMMETRIC AND CHIRAL ORGANOPHOSPHATES WITH WILD-TYPE AND MUTANT ACETYLCHOLINESTERASES.

Zoran Radić and Palmer Taylor
Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093-0636.

The active serine of acetylcholinesterase (AChE) (EC 3.1.1.7) is readily acylated by organophosphorus compounds (OPs) rendering the enzyme inactive. OPs employed as pesticides or of potential concern as chemical warfare agents typically bear no charge and inhibit the enzyme at rates slower than cationic OPs. The inhibition rates for both cationic and neutral OPs are reduced by most ligands binding reversibly at the active center. We have measured rates of inhibition of mouse AChE by paraoxon, DDVP, and enantiomers of neutral alkyl methylphosphonyl thioates and cationic alkyl methylphosphonyl thiocholines in the presence and absence of AChE peripheral site inhibitors: gallamine, *d*-tubocurarine, propidium, atropine and derivatives of coumarin. All ligands, except the coumarins, at submillimolar concentrations enhanced the rates of inhibition by neutral OPs while inhibition by cationic OPs was slowed down. The coumarins slowed down inhibition rates for both neutral and charged OPs. The magnitude of the enhancement ranged from two-fold to more than an order of magnitude. When extended to millimolar concentrations, peripheral site ligands slowed down the inhibition rates by neutral OPs creating a bell shaped activation profile.

The magnitude of enhancement of the inhibition rates was reduced by amino acid replacements at the peripheral site, whereas selective replacements at the choline binding subsite increased the magnitude of enhancement of inhibition achieved with peripheral site ligands.

These observations suggest that peripheral site ligands are capable of allosterically affecting conformation of residues in the choline binding site of AChE thus optimizing the position of the leaving group of uncharged OPs during the inhibition reaction.

L 09

REVERSIBLE INHIBITION OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE BY A COUMARIN DERIVATIVE AND BY 4,4´-BIPYRIDINE

V. Simeon-Rudolf, Z. Kovarik, *Z. Radić and E. Reiner

*Institute for Medical Research and Occupational Health, P.O.Box 291, 10001 Zagreb, Croatia, *Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093-0636, USA*

It was shown earlier that the coumarin derivative 3-chloro-7-hydroxy-4-methylcoumarin (CHMC) is an allosteric inhibitor of acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; 3.1.1.8) while 4,4'-bipyridine (4,4´-BP) binds to both the catalytic and allosteric site of the enzyme. Binding of the CHMC and 4,4'-BP to recombinant mouse w.t. AChE and BChE, to peripheral site-directed AChE mutants and to human serum BChE variants has now been studied. The enzyme activity was measured with acetylthiocholine as substrate. Enzyme/inhibitor dissociation constants for the catalytic and peripheral sites were evaluated from the apparent dissociation constants as a function of the substrate concentration (0.1 -10 mM).

The competition between substrate and CHMC displayed two binding sites on AChE mutants: Y72N, Y124Q, W286A and W286R, and the atypical and fluoride-resistant BChE variants. The dissociation constants for the peripheral site (from 0.06 to 0.1 mM) were on average two times higher than for the catalytic site (0.02 - 0.06 mM). CHMC displayed binding only to the catalytic site (K_a = 0.1 mM) of Y72N/Y124Q/W286A mutant and only to the peripheral site of AChE w.t. (K_i = 0.03 mM) and usual BChE (K_i = 0.2 mM). 4,4´-BP bound to both sites of the AChE mutant Y72N/Y124Q/W286A, AChE w.t. and BChE w.t.. The two compounds had the lowest affinity for the Y72N/Y124Q/W286A mutant and displayed the most pronounced competition with acetylthiocholine over the studied substrate concentration range. However, the K_m constant of the mutant was similar to those of the other enzymes. The enhancement of competition might therefore be attributed, in part, to the differential effect of mutations on the binding of the substrate and the compounds CHMC or 4,4'-BP.

L 10

Acetylcholinesterase knockout mouse, butyrylcholinesterase tetramers, and paraoxonase active site. Oksana Lockridge
Eppley Institute, Univ.Nebraska Med.Ctr., 600 S. 42nd St., Omaha, NE, USA

We are in the process of making the acetylcholinesterase knockout mouse. The aim of these studies is to determine what role, if any, the AChE protein plays in embryonic development. We have succeeded in making chimeric mice that carry the knocked out ACHE gene. These mice are being bred to determine if they are able to transmit the deleted ACHE gene to their offspring. We anticipate that the heterozygous knockout, in which only one allele has been knocked out, will live to birth and adulthood. However, the homozygous knockout is expected to die during embryogenesis. See poster by Xie et al.

Another question we are studying is how to make tetramers of BChE. Tetramers are desired because they are more stable than dimers and monomers. Human BChE expressed in CHO cells consists primarily of dimers and monomers and only 15% tetramers. The percent tetramers increased to 70% when polyproline was added to the culture medium, a result first reported by Bon et al. for AChE (J. Biol. Chem. 1997, 272:3016-21). The polyproline seems to act through the conserved 7 aromatic amino acids located at the C-terminus, since substitution of these 7 aromatic residues resulted in the complete absence of tetramers, despite the presence of polyproline. See poster by Altamirano et al.

A third enzyme we are studying is human paraoxonase. Our goal is to identify the active site of paraoxonase. We used site-directed mutagenesis and transient expression in 293T cells to identify amino acids essential for activity. Eight mutants had no significant activity. They were E52A, D53A, H114N, H133N, H154N, H242N, W280A, and H284N. We expect that some of these amino acids participate in calcium binding and others in catalysis. See two posters by Josse et al.

Supported by U.S. Army Medical Research and Materiel Command DAMD17-94-J-4005, DAMD17-97-1-7349, Association Francaise Contre les Myopathies (MNM1997), and Nebraska State Research Initiative.

L 11

DESIGN AND CONSTRUCTION OF BUTYRYLCHOLINESTERASE MUTANTS THAT HAVE ORGANOPHOSPHORUS ACID ANHYDRIDE HYDROLASE ACTIVITY

C.A. Broomfield, 0. Lockridge and C.B. Millard. U.S. Army Medical Research Institute of Chemical Defense, APG, MD 2I0I0-5425 and University of Nebraska Medical Center, Eppley Cancer Institute, Omaha, NE 68198-6805.

Our goal is to design, express and characterize mutants of cholinesterases that resist or hydrolyze the organophosphorous (OP) nerve agents. Our initial studies have been concentrated on human serum butyrylcholinesterase (BuChE; EC 3.I.I.8) because of its relatively open active site region. By computer-aided molecular modeling based upon the crystal structure of acetylcholinesterase, several residues were selected for site-specific replacement with histidine. We reasoned that introducing an appropriately positioned imidazole group could promote general base catalysis to hydrolyze the phosphylated active site serine. The approach was oligonucleotide-directed mutagenesis in M13mp19 and subsequent stable expression in both CHO and human 293 cells by using a cytomegalovirus promoter and the geneticin drug resistance gene. One of the histidine mutants, G117H, was found to retain butyrylthiocholine (BuSCh), acetylthiocholine and benzoylcholine (Bz) activity at pH 7.4 with a K_{m2}=0.23±O.O17 mM for BuSCh. Wild type, recombinant BuChE had a K_{m2}=0.20±O.O16 mM for BuSCh. Using BuSCh to measure activity, we found that the inhibition rates for the BuChE G117H mutant were markedly decreased for soman, sarin, tabun, DFP, echothiophate (EcSH) and VX. For soman, sarin and DFP inhibition, wild type BuChE has k_i values in the range of 20,000 $M^{-1}sec^{-1}$; for VX the k_i is 30,400 $M^{-1}sec^{-1}$. However, for G117H the k_i was 2.8 $M^{-1}sec^{-1}$ for sarin and 26.4 $M^{-1}sec^{-1}$ for VX. GB and VX-inhibited G117H undergo (relatively) rapid spontaneous reactivation with k_{max} values of 6.8 x 10^{-5} and 16.3 x 10^{-5} sec^{-1}, respectively. This reactivation constitutes hydrolysis of these nerve agents and represents a rate enhancement of 100- and 2000-fold for GB and VX, respectively, at pH 6.0. Comparison of these kinetic data with those of G117K indicates that the observed characteristics of G117H are not due to the presence of a positive charge at the 117 position. Insertion of a second mutation, E197Q, results in an enzyme that also catalyzes the hydrolysis of soman. Inhibition studies of these mutants with carbamates leads to the conclusion that slow reaction with OPs is due to weakening of transition state stabilization by the insertion of histidine into the oxyanion hole.

L 12

STEREOCHEMICAL CONSTRAINTS ON THE HYDROLYSIS OF ORGANOSPHOSPHATE NERVE AGENTS BY PHOSPHOTRIESTERASE.

Frank M. Raushel, Department of Chemistry, Texas A&M University, College Station, Texas USA

The phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of organophosphate nerve agents with inversion of stereochemistry at the phosphorus center. The kinetic constants for the hydrolysis of paraoxon (diethyl p-nitrophenyl phosphate) are approximately 8,000 s^{-1} and 5 x 10^7 $M^{-1}s^{-1}$ for k_{cat} and k_{cat}/K_m, respectively. The effects on the catalytic constants induced by changing the size of the two non-leaving group substituents attached directly to the phosphorus center have been measured for methyl, ethyl, isopropyl, and phenyl groups. There is a significant increase in K_m when the two ethyl group of paraoxon are changed to methyl substituents and a significant drop in k_{cat} when these same groups are changed to isopropyl. Kinetic studies with mixed substituents indicate that there is a significant catalytic preference for one stereoisomer over the other. These studies demonstrate that phosphotriesterase can be used quite effectively for the chiral resolution of racemic mixtures of organophosphate nerve agents.

L 13

Rational Design of Organophosphate Hydrolase for Altered Substrate Specificities

Barbara diSioudi[1], Charles L. Miller[1], Kaihua Lai[1], Leonardo Scapozza[1,2],Janet K. Grimsley[1], and James R. Wild[1]
[1]Department of Biochemistry & Biophysics, Texas A&M University System,College Station, Texas, USA 77843-2128
[2]Department of Pharmacology, ETH, Zurich, Switzerland

Organophosphorus Hydrolase (OPH) is a bacterial enzyme that hydrolyzes a variety of OP neurotoxins, including many widely used pesticides and chemical warfare agents which contain various P-O, P-F, P-CN and P-S bonds capable of inhibiting acetyl-cholinesterase, butyryl-cholinesterase, and/or neurotoxic esterase. OPH has extremely high efficiency in hydrolysis of many different phosphotriester and phosphothiolester pesticides (kcat = 50 to 5,000 s^{-1}) as well as phosphorofluorates such as DPF and the chemical warfare agent Sarin (kcat = 50 to 500 s^{-1}). In contrast, the enzyme has much lower catalytic capabilities for phosphonothioate neurotoxins such as acephate (kcat = 20) or the chemical warfare agent VX [O-ethyl S-(2-diisopropyl aminoethyl) methyl phosphonothioate] (kcat = 0.3 s^{-1}). Different metal-associated forms of the enzyme demonstrated significantly varying hydrolytic capabilities for VX and its analogues, and the activity of OPH (Co+2) was consistently higher than that of OPH (Zn+2) by five to twenty fold. Recent studies have shown that the enzyme is approximately four-fold better at the destruction of the Russian o-isobutyl [O-(2-diethylamino methyl phosphonothioate]. In addition to the metal substitutions, protein engineering modifications have been developed and have resulted in significant enhancement of the rates of detoxification of the thioate pesticides and chemical warfare agents. In order to develop a series of applied interests, it has been necessary to remove, modify, substitute, and add to native leader polypeptide sequences in order to stabilize the protein as well as to direct the enzyme to various cellular locations. Modeling studies based on the structures of H. Holden et al. have produced a molecular insight to the structural changes accounting for these kinetic alterations.

L 14

PARAOXONASE (*Pon1*) POLYMORPHISM - WHAT'S IMPORTANT, GENOTYPE, PHENOTYPE OR BOTH?

C.E. Furlong[1], R. J. Richter[1], W.F. Li,[2] and L.G. Costa[2], Depts. of Medicine[1] and Environmental Health[2] University of Washington, Seattle, WA

In human populations, serum paraoxonase (PON1) exhibits a substrate dependent polymorphism. The Arg_{192} isoform hydrolyzes paraoxon rapidly but diazoxon, soman and especially sarin slowly. On the other hand, the Gln_{192} isoform hydrolyzes paraoxon slowly, but diazoxon, soman and sarin more rapidly than the Arg_{192} isoform. Our experiments with a mouse model system have convincingly shown that PON1 plays a major role in the detoxication of OP compounds processed through the P450/Ponl pathway (see Costa *et al.*). Recent studies have also shown that PON1 plays an important role in the metabolism of oxidized lipid compounds (Watson *et al.*, J. Clin. Invest. 96:2882). Currently, there is an effort underway to identify genes and polymorphisms that play an important role in "environmental susceptibility". The *Pon1* polymorphism has been cited as a prime example of such a genetic polymorphism (Science 278:569). The advent of the polymerase chain reaction (PCR) for DNA amplification with improvements, modifications and automation has provided a very convenient way to do individual genotyping. It is tempting to set up large scale PCR analyses of populations to determine individuals at risk for environmental exposures affected by the specific polymorphism. In fact, a number of such studies have already been carried out in examining the relationship of the *Pon1* polymorphism to vascular disease. We describe a high throughput two-dimensional enzyme assay that provides both *Pon1* genotype and phenotype (PON1 status). The high level of variation of gene expression within each genetic class in humans, together with our animal model studies indicate that it is very important to determine PONl status as opposed *to Pon1* genotype alone. (Supp. in part by grants ES 05194 and 07033)

L 15

ON THE PHYSIOLOGICAL ROLE(S) OF THE PARAOXONASES

Bert N. La Du[1], Michael Aviram[2], Scott Billecke[1], Mohamad Navab[3], Sergio L. Primo-Parmo[1], Robert C. Sorenson[1], and Theodore J. Standiford[4]

[1]Departments of Pharmacology and Anesthesiology, University of Michigan Medical School, Ann Arbor, MI, 48109; [2]Lipid Research Laboratory, Rambam Medical Center and the Bruce Rappaport Faculty of Medicine, Technion and the Rappaport Family Institute for Research in the Medical, Sciences, Haifa Israel 31096. [3]Division of Cardiology, Department of Medicine School of Medicine University of California, Los Angeles, CA90095; [4]Department of Medicine, University of Michigan Medical School, Ann Arbor, MI 48109.

In recent years several lines of evidence have indicated that serum paraoxonase (PON1), and perhaps the other mammalian paraoxonases, act as important guardians against cellular damage from agents such as the oxidized lipids in low density lipoproteins (Ox-LDL), as well as from bacterial endotoxins. These protective properties of the enzyme may not need calcium ions, the specific metal required for its hydrolytic activity with paraoxon and phenyl acetate. Broadening of the paraoxonase catalytic activities also means that its protective roles are not necessarily only hydrolytic, and hence an investigation of other potential functions is under way.

The zinc form of human serum PON1 protects against LDL oxidation, but the zinc form is unable to hydrolyze either paraoxon or phenyl acetate. Furthermore, the cysteine at position 293 is required for protection against LDL oxidation, even though when this residue is replaced by either alanine or serine the resulting mutants are still able to hydrolyze phenyl acetate and paraoxon. Additional studies with other structural mutants support the conclusion that the specific structural requirements for aryl esterase/ paraoxonase activity and for protection against oxidative damage are different, although all the allozymes of PONI participate in both types of reactions. The localization of PON1 within the high density lipoprotein (HDL) fraction of serum probably largely accounts for the previously noted protective effects of high concentrations of serum HDL levels against cardiovascular disease, and against endotoxin toxicity. The mechanism by which HDL-associated PON1 exerts these beneficial effects needs still to be determined.

L 16

PARAOXONASE (Pon1) AND SENSITIVITY TO ORGANOPHOSPHATE TOXICITY: ANIMAL STUDIES

L.G. Costa[1], W.F. Li[1], R. J. Richter[2], A. Lusis[3], D. M. Shih[3], C.E. Furlong[2], Depts. of Environmental Health[1] and Medicine[2], University of Washington, Seattle, WA; Dept. of Medicine, UCLA, Los Angeles, CA.[3].

Based on the polymorphism of Pon1 in human populations and the known role of A-esterase in detoxifying the active metabolites of several organophosphates (OPs), it has been inferred for some time that the expression of this enzyme plays an important role in determining susceptibility to OPs. Yet, direct proof of this hypothesis has been obtained only in the past few years by a number of animal studies that are here summarized. Experiments in rats and mice have shown that injection of Pon1 purified from rabbit serum by the iv, im or sc route, significantly increases Pon1 activities in rodent's plasma. Under these conditions, the acute toxicity (assessed by the degree of acetylcholinesterase inhibition) of paraoxon and chlorpyrifos oxon is significantly decreased, compared to control animals. Protection is maximal when Ponl is administered before the OPs, but still occurs when Pon1 is utilized as a post-exposure treatment. Furthermore, protection by Pon1 is also provided toward the parent compound chlorpyrifos. Pon1-knockout mice display a much greater sensitivity to chlorpyrifos oxon toxicity than wild mice. However, the acute toxicity of guthion, which is not a substrate for Pon1, does not differ between knockout and wild mice. Studies in mice show that treatments with 3 methyl cholanthrene, β-naphtoflavone, phenobarbital, dexamethasone and endotoxin does not affect, or decrease, plasma Pon1 activity. Young animals are known to be more sensitive to the acute toxicity of OPs than adults, and this may be due to a low expression of Pon1. Indeed, Pon1 mRNA in liver and Pon1 activity in plasma increase with age in mice and rats reaching a peak around postnatal week 3 or 4. Altogether, these data provide strong evidence that Pon1 activity is a major determinant of the susceptibility to acute toxicity for substrate OPs. Whether this would still be a valid assumption in case of prolonged, low-level OP exposure, remains, however, to be determined. (Supp. in part by ES-07033, ES-05104, & PO1HL30568).

L 17

Organophosphorus Acid Anhydrolase (OPAA) in Slime Mold, Duckweed and Mung Bean: a Continuing Search for a Physiological Role and a Natural Substrate

Francis C.G. Hoskin, Marine Biological Laboratory, Woods Hole, MA, USA 02543, **and John E. Walker and Charlene M. Mello**, U.S. Army Natick RD&E Center, Natick, MA, USA 01760

Recently, and for the first time, a DFP-hydrolyzing enzyme, i.e., an OPAA, has been reported in a plant source (Yu and Sakurai, Environ. Sci. 3, 103-111 (1995)). Based on this and other suggestive evidence, we have tested the ability of two plants and a protist to hydrolyze DFP and Soman, and the effects of Mn^{2+} on this activity. The plants are a particular strain of duckweed *(Lemna minor),* and germinated mung bean *(Vigna radiata)*; the protist is a slime mold *(Dictiostylium discoidium).* The tests are based on a crude classification of OPAAs as "squid type" (DFP hydrolyzed more rapidly than Soman; no Mn^{2+} stimulation) and OPAAs from all other sources (Soman hydrolyzed more rapidly than DFP; several-fold Mn^{2+} stimulation as first described by Mazur). We find that while both duckweed and mung bean hydrolyze Soman and the hydrolysis is Mn^{2+} stimulated, neither source hydrolyzes DFP, either with or without Mn^{2+}. Also in marked contrast to the report by Yu and Sakurai, Soman hydrolysis by mung bean is completely inhibited by EDTA. The slime mold hydrolyzes Soman more rapidly than DFP (but does hydrolyze DFP) and the hydrolysis is Mn^{2+} stimulated. The failure of either of these plant sources to hydrolyze DFP is similar to the behavior of OPAA from *Bacillus stearothermophilus*, reported at the first Dubrovnik meeting in 1988. This is part of a continuing search for a natural substrate and a physiological role for either of these major OPAA classifications.

L 18

IMPROVEMENTS IN SCAVENGER PROTECTION AGAINST ORGANOPHOSPHORUS AGENTS BY MODIFICATION OF CHOLINESTERASES.

Donald M. Maxwell[1], Ashima Saxena[2], Alan J. Russell[3], and B. P. Doctor[2]. [1]U. S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, [2]Walter Reed Army Institute of Research, Washington, DC, [3]Center for Biotechnology and Bioengineering, University of Pittsburgh, PA. (USA)

The effectiveness of stoichiometric scavengers, such as cholinesterases (ChEs), to protect against a variety of organophosphorus (OP) agents has been demonstrated in several *in vivo* models. A variety of approaches have been used to improve the capability of ChEs to scavenge OPs. The *in vitro* stoichiometric neutralization of sarin by acetylcholinesterase (AChE) was increased from 1:1 to 3200:1 by the addition of 2 mM HI-6 oxime, while the *in vivo* stoichiametry was increased to 57:1 in mice by HI-6. The aging rate of soman-inhibited mouse (Mo) AChE at pH 7.3 was reduced 23-fold in a mutant Mo-AChE ($E_{202}Q$) where glutamate was replaced by glutamine, resulting in a 2-fold increase in oxime-assisted neutralization of soman. To improve the duration of scavenger protection provided by ChEs, the mean residence times of five tissue-derived and two recombinant ChEs injected i.v. in mice were compared with their oligosaccharide profiles. Although sialylation was essential for maintaining ChEs in circulation, these studies revealed that the position of sialylated galactose residues may be more important than the extent of sialylation in determining circulatory half-life. The stability of AChE in non-physiological environments was improved by immobilizing it in a polyurethane foam matrix that allowed AChE to retain activity at high temperature (80 °C) where native soluble enzyme denatured. These developments in scavenger technology have improved the *in vivo* protection provided by OP scavengers and extended their applicability to provide external decontamination of chemical agents and pesticides.

L 19

PROMOTION OF AXONOPATHY BY CERTAIN ESTERASE INHIBITORS

Marcello Lotti, Università degli Studi di Padova, Istituto di Medicina del Lavoro, Via Giustiniani 2, I-35128, Padova, Italy.

Certain esterase inhibitors, including organophosphates, organophosphinates, sulfonylhalides, carbamates and thiocarbamates, elicit or intensify the clinical expression of various insults to axons. This phenomenon was called promotion of axonopathies because these chemicals are not additive neurotoxicants nor do they interfere with the pharmacokinetics. Characterization of promotion was carried out by using organophosphate induced delayed polyneuropathy (OPIDP) as a model. Consequently it was found that all promoters are inhibitors of Neuropathy Target Esterase (NTE, the molecular target for OPIDP), although the phenomenon is not correlated with NTE inhibition. Another esterase was identified which is being characterized in nervous tissues and is inhibited when promotion occurs. The search for a physiological explanation of promotion has the following background: 1. Promotion expresses clinically the biochemical lesions which are otherwise well compensated (such as 30/40% NTE inhibition by neuropathic organophosphates). 2. Promotion is not specific because axonopathies of different origin are affected. 3. Promoters are effective when given several days before the neuropathic insult. 4. Promotion is less effective in young animals as compared with adults. 5. Promotion occurs when axons, but not necessarily the cell body, are targeted by promoters. 6. Repeated dosing with a promoter failed to produce axonopathy. Based on this evidence it is suggested that promotion might interfere with a mechanism(s) of compensation and/or repair of long axons.

L 20

THE SEARCH FOR THE TARGET OF PROMOTION OF AXONOPATHIES. Angelo Moretto, Istituto di Medicina del Lavoro, Università degli Studi di Padova, via Giustiniani 2, I-35128 Padova, Italy

The target of promotion of axonopathies is thought to be similar or linked to neuropathy target esterase (NTE), the target for organophosphate induced delayed polyneuropathy (OPIDP). NTE is defined as the phenyl valerate esterase activity (PVE) in nervous tissues resistant to paraoxon and sensitive to mipafox (40 μM and 50 μM, pH 8.0, 20 min, respectively). Mipafox (50 μM) resistant PVEs include some activity sensitive to the promoter phenylmethane sulfonylfluoride (PMSF) but no correlation was found between its inhibition and promotion. It was concluded that the target of promotion was not a PVE other than NTE. However, a complete titration curve of paraoxon-resistant PVEs by mipafox (0-1 mM) dissected, besides NTE (I_{50} about 7 μM), another PVE with an I_{50} of approximately 200 μM (M200). Therefore, M200 was operationally defined as the PVE resistant to paraoxon and mipafox (40 μM and 50 μM, respectively) and sensitive to mipafox (1 mM). M200 was present in hen brain, spinal cord and peripheral nerve, corresponding to about 10, 20 and 30% of NTE activity, respectively. M200 I_{50}s in nervous tissues for the neuropathic compounds diisopropylfluorophosphate (DFP) and dibutyldichlorvos were in the range of 2-10 and 0.06-0.2 μM respectively. Corresponding NTE I_{50} were in the range of 0.2-0.5 and 0.004-0.008 μM, respectively. The I_{50} for promoters PMSF (about 100 μM) and butylsulfonyl fluoride (about 60 μM) were similar for both NTE and M200. In vivo data showed, among others, selective inhibition of M200 by the promoter KBR 2822 (O-(2-chloro-2,3,-trifluorocyclobutyl)O-ethyl S-propyl ester) and of NTE by DFP. This suggested that M200 inhibition correlates with promotion. Two forms of different molecular weights of mipafox-sensitive PVEs have been identified by means of chromatography in the supernatant of peripheral nerve which might correspond to NTE and M200.

L 21

NTE SOLUBLE ISOFORMS: new perspectives for targets of neuropathy inducers and promoters

Eugenio Vilanova, Maria Angeles Escudero, José Barril
Universidad "Miguel Hernández". Campus de San Juan (Alicante). Spain.

The brain membrane bound (particulate) PVase defined by Johnson in 1969 as NTE and recently cloned by Glynn et al. we call it here as P-NTE. It is related with the OP delayed neuropathy and it is the activity measured in standard NTE assays. Following the same operational criteria in soluble fraction of sciatic nerve, paraoxon-resistant mipafox-sensitive PVase was described and defined as S-NTE (soluble-NTE) with apparent lower sensitivity to some inhibitors than P-NTE. Two isoforms (S-NTE1 and S-NTE2) were separated. In partly purified S-NTE2 preparation, polypeptides were identified in western blots by labelling with the same bitinylated inhibitor S9B used to label and isolate P-NTE but it was not visualised with anti-P-NTE antibodies. From studies of sequential inhibition, reversibility and time course inhibition, it is deduced that the reversibility of inhibition is a new factor introducing a higher complex situation in identifying the esterases that could be candidates as targets of the mechanisms of induction or promotion of neuropathy and that all the P- and S-NTE assay protocols will need to be reviewed. Data published by M. Lotti, A. Moreto et al suggest that P-NTE cannot be the target for promotion. The proposal that S-NTE2 could be the candidate to promotion target is suggestive and under collaborative biochemical and toxicological studies. Work partly supported by grants FIS-95.0053 and a grant to JB by Generalitat Valenciana.

L 22

NOVEL PROTEIN TARGETS FOR ORGANOPHOPHORUS COMPOUNDS.

Paul Richards, Colin Walker[1], David Ray, Martin Johnson.
Neurotoxicology Group, MRC Toxicology Unit. University of Leicester, Leicester LE2 3ER.
[1]School of Animal and Microbial Sciences, University of Reading, Reading, RG6 6AJ.

In recent years concern has been expressed that not all the toxic effects of OPs can be ascribed to their reaction with known targets. In particular the putative long-term health effects as a result of chronic low-level exposure appear to be unrelated to previously described effects of OP poisoning. To address this issue we are currently screening nervous tissue for the existence of sites more sensitive to particular OPs than acetylcholinesterse in order to find novel protein targets. Focus has initially centred on two brain proteins (molecular masses 30 and 85kDa) which have been shown to have differential sensitivity to a range of OP compounds. We have characterised the 85kDa protein and shown it to be identical to *N*-acylpeptide hydrolase, an enzyme of unknown biological function but with a significant esterase activity (butyrylesterase). *N*-acylpeptide hydrolase will catalyse the removal of *N*-acylated amino acids from the *N*-terminal of short peptides and it has been speculated that this function is important in the catabolism of growth factors and peptide hormones. The enzyme is particularly sensitive to the pesticide dichlorvos (2,2-dichlorovinyl dimethylphosphate) both *in vitro* and *in vivo*. Furthermore, *N*-acylpeptide hydrolase does not appear to reactivate (unlike acetylcholinesterase) and can be significantly inhibited for several days following a single dose of dichlorvos.

L 23

Molecular Cloning of Neuropathy Target Esterase (NTE).

P. Glynn, D.J. Read, M.J. Lush, J.M. Atkins and Y. Li,
MRC Toxicology Unit, University of Leicester, Leicester LEl 9NH, UK.

Modification of NTE, a neuronal protein with serine esterase activity, by certain organophosphates (OP) initiates degeneration of long axons in the peripheral and central nervous system. To elucidate the roles of NTE in neuronal function and in OP-induced neuropathy we have isolated this protein and cloned its cDNA. NTE was affinity-purified from pig brain tissue using the biotinylated OP reagent S9B, digested with V8 protease and the N-terminal sequence of several electrophoretically-resolved fragments was determined (Glynn *et al*, 1994; Biochem. J. **301**, 551-556). One peptide sequence was found to be homologous to a human expressed sequence tag and the latter was used to isolate a partial NTE clone (2.2 kb) from a human cDNA library. This clone was used to probe Northern blots and hybridized to transcripts of 4.5-5 kb in brain, kidney, skeletal muscle and other tissues. A full-length cDNA clone was subsequently isolated from a second human library and was subcloned into a mammalian expression vector. Portions of the NTE cDNA encoding its putative enzyme domain were cloned into a pET vector for expression in bacteria. The deduced amino acid sequence of NTE contained all the peptides determined by Edman degradation and holds important clues regarding the mechanism of OP-induced neuropathy.

L 24

CHLORPYRIFOS-OXON IS A RAPID INHIBITOR OF BChE

G. Amitai†*, D. Moorad*, R. Adani†, and B.P. Doctor*. *Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA and † Israel Institute for Biological Research, 74100 Ness-Ziona, Israel

Phosphorothionate insecticides such as parathion and chlorpyrifos (CPS, Dursban) are metabolically converted by oxidative desulfuration into paraoxon and chlorpyrifos-oxon (CPO).We have examined the kinetics of inhibition of AChE and BChE by paraoxon and CPO. Bimolecular rate constants (k_i) for inhibition by paraoxon of recombinant human (rH) AChE, recombinant mouse (rM) AChE and fetal bovine serum (FBS) AChE are 7.0, 4.0 and 3.2 x 10^5 M^{-1} min^{-1}, respectively. The k_i values for the inhibition by CPO of rH AChE, FBS AChE, human RBC AChE, *Torpedo* AChE, and rM AChE are 9.3, 2.2, 3.8, 8.0, and 5.1 x 10^6 M^{-1} min^{-1}, respectively. Inhibition of human serum BChE, rH BChE, and rM BChE by CPO yielded k_i values of 1.65, 1.67, and 0.78 x 10^9 $M^{-1}min^{-1}$, respectively. Due to the rapid kinetics of BChE inhibition by CPO it was followed in real-time in the presence of substrate. The k_i values obtained for BChE from various species are 160-750 fold larger than those of AChE from parallel sources. Inhibition of the single-site mutant $A_{328}Y$ of rH BChE by CPO displayed a 21-fold lower rate than that of wild-type rH BChE (k_i, 7.9 x 10^7 vs. 1.67 x 10^9 M^{-1} min^{-1}). The double mutant of acyl pocket residues of rH AChE, $F_{295}L/F_{297}V$, was inhibited by CPO with a 150-fold larger k_i than wild type (1.5 x 10^9 vs. 1.0 x 10^7 M^{-1} min^{-1}). The increased rate obtained with the double mutant rH AChE displaying characteristics of BChE active site and the slower rate obtained with the $A_{328}Y$ BChE mutein provides a rationale for higher efficacy of CPO scavenging by BChE, compared to AChE.

L 25

Inhibition of Drosophila Acetylcholinesterase by 7-(methylethoxyphosphinyloxy) 1-methyl-quinolinium iodide (MEPQ)

Veronique Marcel[1], Didier Fournier[1] and Jure Stojan[2]

[1]Laboratoire d 'Entomologie et Groupe de Chimie Biologie, Universite Paul Sabatier, 31062 Toulouse, France.

[2]Institute of Biochemistry, Medical faculty, University of Ljubljana, 1000 Ljubljana, Slovenia.

Insect acetylcholinesterase displays activation at low substrate concentrations and inhibition at high substrate concentrations. A model which is able to explain the kinetic behaviour of all cholinesterases and also the double character of this enzyme has been suggested recently.

In order to obtain further information on the molecular events taking place during the catalytic action of insect acetylcholinesterase, the hydrolysis of acetylthiocholine in the presence of different concentrations of MEPQ was performed. The reaction at low substrate concentrations was followed until the change of the absorbance became negligible and at high concentrations only initial parts were recorded. A simultaneous analysis of the progress curves using numerical integration treatment which takes into account the depletion of all reactants during the reaction showed, that relatively fast acting synthetic organophosphorous compound binds and compete with the substrate for the same binding sites. According to the model and the determined kinetic parameters the same molecular evens take place during the phosphorylation process as during the acylation of the enzyme by the substrate. The results are also in accordance with the thesis that every molecule that enters into the active site gorge is metabolized.

L 26

Antiatherogenic Properties of Human Serum Paraoxonase (PON)

M.I. Mackness
University Department of Medicine, Manchester Royal Infirmary, Manchester M13 9WL UK

The oxidative modification of low-density lipoprotein (LDL) is central to the pathogenesis of atherosclerosis. High-density lipoprotein (HDL) is known to protect against the development of atherosclerosis, however, the exact mechanism remains unclear. The role of HDL in reverse-cholesterol transport was believed to be responsible for its protective action. Recent findings from several laboratories showing that HDL can prevent LDL oxidation suggest an additional mechanism.

PON which is solely associated with HDL was first shown to us to prevent LDL oxidation by Cu^{2+} or macrophages by decreasing lipid-peroxides on LDL. The PON alloenzymes differ in their ability to prevent LDL oxidation which may explain some of the recent epidemiological studies which show PON genotvpe as an independent risk factor for CAD. PON may act in concert with other HDL enzymes such as PAF-AH and LCAT providing an efficient mechanism to protect LDL against oxidation in the artery wall as atherosclerosis progresses provides further evidence for a role for PON in preventing lipoprotein (and cell membrane) lipid-peroxidation.

L 27

ORGANOPHOSPHORUS HYDROLASES AS ANTIDOTES AGAINST ORGANOPHOSPHATES TOXICITY: A PHOSPHORYLATED OXIME IS SUBSTRATE OF BACTERIAL PHOSPHOTRIESTERASE

Haim Leader[‡], Nathan Rothschild[§], Carlos Dosoretz[§] and Yacov Ashani[‡],

Israel Institute for Biological Research[‡], P.O. Box 19, Ness-Ziona, Israel, and MIGAL-Galilee Technological Center[§], Kiryat Shmona, Israel

Organophosphorus hydrolases (OPH; Phosphotriesterases) from *Pseudomonas* sp. and *Pseudomonas diminuta,* were shown to protect mice against organophosphates (OP) toxicity by hydrolyzing circulating OPs. Recently, an OPH purified from *Pseudomonas* sp. has been demonstrated to hydrolyze quaternary ammonium-containing OPs such as 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ), and *O,O*-diethylphosphinyloxy-1- methylquinolinium methyl sulfate (DEPQ; k_{cat}/K_m = $3.0x10^8$ $M^{-1}min^{-1}$). Oxime-induced reactivations of OP-cholinesterase conjugates (OP-ChE) are believed to be accompanied by formation of potent anti-ChE phosphorylated oximes (POX). Since POXs contain quaternary ammonium aromatic poles it was hypothesized that OPHs might effectively detoxify them, as was obseived with MEPQ and DEPQ. Rapid detoxification of POXs is important for minimizing re-inhibition of reactivated ChEs. To examine such potentiality, a POX that is assumed to accumulates during reactivation of *O,O*-diethylphosphoryl-ChE by 4-(hydroximinomethyl)-1-methylpyridinium iodide (4-PAM), was synthesized and characterized as a ChE inhibitor and OPH substrate. *O,O*-diethlyphosphoryalted 4-PAM (DEP-POX) is a prototype of POXs derived from reactivators such as TMB_4 and toxogonin. DEP-POX was found a potent ChE inhibitor, with the following ranking order: HuBChE>> EqBChE> MoAChE> FBS-AChE. k_{cat}/K_m of OPH-induced hydrolysis of DEP-POX approached $3.6x10^7$ $M^{-1}min^{-1}$ at k_{cat} = 28,000/min. Based on *in vitro* studies, it is suggested that OPHs can enhance oxime-induced reactivation via the rapid hydrolysis of POXs. These observations further substantiate the claim that treatment with a combination of OPH and a suitable oxime may significantly improve therapeutic management of OP toxicity.

L 28

AChE IN THE NEUROMUSCULAR JUNCTION

Janez Sketelj, Neva Črne-Finderle
Institute of Pathophysiology, University of Ljubljana, Slovenia

Acetylcholinesterase (AChE) is higly concentrated in the neuromuscular junctions (nmjs) of vertebrates. Its location remains stable after denervation in mature muscles but not in early postnatal muscles. Synaptic basal lamina contains agrin which is able to induce a sarcolemmal differentiation mimicking the postsynaptic membrane even in the absence of a nerve ending. AChE in the nmjs in mammals is largely present as the asymmetric molecular form consisting of a head with up to 12 catalytic subunits, and a collagenous tail. Activity of these forms drops rapidly after denervation, but decreases slowly in paralyzed muscles although in both AChE synthesis is greatly depressed. Activity of the junctional asymmetric AChE forms depends also on the pattern of muscle activation so that their activity is lower in the nmjs of slow than those of fast rat muscles. The tail of the asymmetric AChE forms is a special collagen protein (collagen Q), its c-DNA has been cloned and sequenced. A larger part of the asymmetric AChE forms in the rat nmj is bound to the basal lamina by electrostatic interactions, but about 30-40% of junctional AChE activity is more firmly bound. The asymmetric AChE forms, however, are not specific for the nmj. In immature postnatal rat muscles, they are present all along the muscle fibers. In fast rat muscles, these AChE forms virtually disappear from the extrajunctional regions during maturation, but they are still produced there in the slow soleus muscle. Moreover, their activity is greatly enhanced during reinnervation of this muscle following motor nerve crush.

L 29

CONTROL POINTS OF ACETYLCHOLINESTERASE SYNTHESIS IN THE MAMMALIAN SKELETAL MUSCLE.

[1]K. Zajc-Kreft, [1]T. Marš, [1]M. Brank, [2]A.F. Miranda, and [1]Z. Grubič
[1]Institute of Pathophysiology, School of Medicine, University of Ljubljana, 1000 Ljubljana, Zaloška 4, Slovenia, and [2]Dpt. of Neurology, College of Physicians and Surgeons of Columbia University, New York, NY, USA

Acetylcholinesterase (AChE) expression in the skeletal muscle is regulated at various levels. Aiming to elucidate the mechanisms underlying this regulation, we have used various models and approaches. *In situ* hybridization studies on the *in vitro* innervated human muscle (Grubič et al., *Neuron,* 14: 317-327,1995) supported the proposal of Jasmin et al. *(Neuron* 11: 467-477, 1993) that electromechanical activity downregulates extrajunctional AChE at the mRNA level. However, AChE mRNA is also decreased after denervation of the adult rat muscle, which argues against the downregulating role of electromechanical activity. Studying degradation rate of deproteinated AChE mRNA after its exposure to various subcellular fractions, isolated from the denervated rat SM muscle, we found significantly increased, but relatively unspecific AChE mRNA degradation capacities in these fractions. Increased muscular mRNA degradation capacity could therefore at least partly explain decreased AChE mRNA level after denervation (Zajc-Kreft et al., in preparation). mRNA level is important, but not the only control point of AChE expression. We found that glucocorticoids downregulate AChE synthesis in the adult fast rat muscle at the unchanged AChE mRNA level. Determinations of AChE molecular forms and AChE activities in the specially prepared polysomic fractions, containing early synthesized AChE, led to the conclusion that glucocorticoids exert their effects at the translational or early posttranslational stage of AChE synthesis (Brank et al., *Eur.J.Biochem,* 1997, in print). Myonuclear distribution might also contribute to the regulation of protein expression in the muscle fiber syncitium and was therefore studied in the normal and denervated fast rat muscle.

L 30

Organophosphate inhibition of Acetylcholinesterase induces a molecular/physiological cascade of feedback events leading toward long-term impairments in cholinergic functions.

Daniela Kaufer, Alon Friedman, Shlomo Seidman, Hermona Soreq. *Department of Biological Chemistry, Life Sciences Institute, Hebrew University of Jerusalem Israel*

Mammalian responses to the cholinergic excitation associated with either acute stress or exposure to anti-cholinesterases involve both immediate activation of cholinergic pathways and long lasting structural and functional changes in the central nervous system, with unknown pathways between the two. Moreover, both acute and chronic exposure to cholinesterase inhibitors induce long-term psychopathologies that are strikingly reminiscent of posttraumatic stress disorder. To explore the mechanism(s) underlying long-term changes in cholinergic neurotransmission, we studied transcriptional and post-transcriptional regulation of key cholinergic genes following either acute stress or exposure to anti-cholinesterase drugs and correlated them with changes in hippocampal electrophysiological activity.

Recording of evoked potentials in the CA1 area of the hippocampus from saggital brain slices exposed to the organophosphate DFP revealed a biphasic response: an immediate increase m the amplitude, rate of rise, and paired-pulse facilitation and a delayed phase of depression of the augmented synaptic response. This response was mimicked by the non-hydrolyzable ACh analog carbamylcholine and blocked by the muscarinic antagonist atropine. RT-PCR analyses demonstrated upregulation of the early immediate gene c-fos in these brain slices, followed by drastic reductions in choline acetyl transferase and vesicular acetylcholine transporter mRNAs and concomitant increases in acetylcholinesterase (AChE) mRNA and protein. Both the electrophysiological and transcriptional response were found to depend on neuronal activation and calcium accumulation.

While modulated cholinergic gene expression acts to reduce available ACh and depress excessive cholinergic excitation, the same compensation process may have potentially damaging long-term implications. We observed over 2- fold higher AChE levels in cortex for over 80 hours following acute stress. Therefore, these mice resemble transgenic mice overexpressing brain AChE. However, AChE transgeneic mice suffer delayed cognitive and neuromotor pathologies. Our findings therefore imply a common mechanism for the delayed neuropsychiatric pathologies associated with stress responses and the delayed cognitive symptoms reported for anti-cholinesterase poisoning. Of particular concern is the possibility that feedback processes leading to elevated levels of brain AChE may be similarly associated with low level exposure to common Organophosphorous anti-cholinesterases.

L 31

TOXICOLOGICAL SIGNIFICANCE IN THE CLEAVAGE OF ESTERASE-β-GLUCURONIDASE COMPLEX IN LIVER MICROSOMES BY ORGANOPHOSPHORUS COMPOUNDS

Tetsuo Satoh[1], Satoshi Suzuki[1] , Naoshi Kawai[1], Toshihiro Nakamura[2], and Masakiyo Hosokawa[2]

[1]Biomedical Research Institute, 2802-1, Hiratsuka, Shiroi, Inba, Chiba 270-14, Japan [2]Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263, Japan

Liver microsomal β-glucuronidase is stabilized within the luminal site of the vesicles of rat liver microsomes by complexation with the accessory protein, named egasyn. In the series of our studies on the mammalian carboxylesterases[1)], we have reported that egasyn is identical to RL2 isozyme which is one of the rat liver microsomal carboxylesterases. The subunit molecular weight and plvalue of RL2 are 61kDa and 5.5, respectively. Organophosphorus compounds(OP) caused a rapid dissociation of the egasyn-β-glucuronidase(EG) complex either when administered *in vivo* or when added *in vitro* to cultured rat hepatocytes. Dissociation of EG complex *in vivo* by OP was followed by massive and rapid secretion of β-glucuronidase into plasma. We have used other animal species than rats to compare the extent of the OP-induced release of liver β-glucuronidase and found that there were remarkable species differences in the increase of the β-glucuronidase activities released in plasma. Thus, in terms of the i*n vitro* studies, rats and mice were most sensitive to exposure to OP and monkeys and humans had relatively less sensitivity than rats. Although the reason for species differences remains unclear at present, the increase of β-glucuronidase in plasma after exposure to OP is much more sensitive marker to the acute intoxication of OP than plasma cholinesterase inhibition.

1) T. Satoh and M. Hosokawa: Mammalian Carboxylesterases: From Molecules to Functions. Annu. Rev. Pharmacol. Toxicol. 38, 257-288(1998)

L 32

Estimation of Inhibitory Organophosphates with Purified Pig Liver Carboxylesterase

Eberhard Heymann - University of Osnabrück/Germany

Organophosphates that inhibit acetyl-choline esterase normally also inhibit pig liver carboxylesterase irreversibly. Since this liver esterase is well characterizid and easily accessible, we propose here the use of this enzyme for the estimation of very low concentrations of such organophosphates. Both methods proposed use esterase assays with low affinity substrates, such as acetyl-tyrosine-ethyl ester or butyl acetate.

In the first of these methods, the inhibitor is added to an esterase assay, and the activities before (v_1) and after (v_2) addition of the inhibitor are recorded. The inhibitor concentration (C_I) is obtained by the equation:

$$C_I = \frac{v_1 - v_2}{v_1} C_E$$

where C_E means the molar esterase concentration, which can be obtained from known kinetic data of the enzyme. This very simple method is limited in that C_I must be in the order of 0.1 to 0.9 C_E.

The second method has no such limitation, but it requires more work, because 2 to 5 esterase assays are necessary for one estimation of C_I. In this case, the amount of the ester cleaved has to be plotted against log ($t \cdot C_E \cdot k_2 + 1$), with t = time, k_2 = second order rate constant for the reaction between enzyme and inhibitor. C_E and C_I are equimolar, if a linear plot results.

The theoretical background of these methods is discussed and practical examples for the estimation of Paraxone (order of 0.1 nmoles) are given.

L 33

KINETIC PARAMETERS AND STRUCTURE-FUNCTION DATA RETRIEVAL THROUGH ESTHER, THE DATABASE ON CHOLINESTERASES AND α/β HYDROLASES.

Arnaud Chatonnet, Thierry Hotelier, Xavier Cousin
Institut National de la Recherche Agronomique Differenciation cellulaire et Croissance Place Viala 34060 Montpellier cedex France
chedb@ensam.inra.fr

We developed the ESTHER database (for esterases, α/β hydrolase enzymes and relatives) since 1994. This internet server is dedicated to several aspects of biology of Cholinesterases.

The database is now managed through the ACeDB software. The new development of ESTHER database and server include data about kinetics. The data is organised in classes where a parameter is described by a composed name such as Ki_Paraoxon_F295A_human_acche.

Data for this parameter include links to the inhibitor (plane formula, chemical formula, references) to the enzyme, the mutation if the enzyme has been genetically engineered. If the same parameter has been determined on the same enzyme by other investigators or on another species, the multiple values are presented for comparison with information on the experimental conditions and the references. Queries and tables can be built to recover data on a reduced set of parameters. An exemple of query is: Kcat of electric eel AChE for acetylthiocholine.

The client-server for connexion to ESTHER, as well as stand alone version of aCHEdb for Mac, PC or Unix platform are available by anonymous ftp from ftp.inra.toulouse.fr in the directory /pub/esther. The W3 adress:http://www.ensam.inra.fr/cholinesterase

Supported by Association Francaise contre les Myopathies

L 34

Metabolisation of substrate by invertebrate cholinesterase

Sandino Estrada-Mondaca[1], Didier Fournier[1], Alain Klaebe[1], Frédéric Magné[1], Véronique Marcel[1], Patrick Masson[2], Jure Stojan[3] and François Villatte[1]

[1] *Laboratoire d'entomologie, Université Paul Sabatier, 31062 Toulouse, France. E-mail: fournier@cict.fr; tel/fax: 05.61.55.69.10*
[2] *Centre de Recherches du Service de Santé des Armées, Unité de Biochimie, 24 Avenue des Maquis du Grésivaudan, B.P. 87, 38702 La Tronche Cedex, France.*
[3] *Institute of Biochemistry, Medical Faculty, Ljubljana, Slovenia*

In two invertebrates, Drosophila melanogaster and Caenorhabditis elegans, we found cholinesterases which show substrate activation at low substrate concentrations followed by inhibition at higher concentrations. This triphasic kinetics could be explained by the presence of two enzymes with different kinetic behaviour but separation of the two putative forms was unsuccessfull. More probably, the simultaneous presence of activation and inhibition results from the existence of a single enzyme regulated by the concentration of the substrate. To understand this comportment, we tested different rival kinetic models assuming that there are only two binding sites for the substrate. Results obtained for different substrates, under differents conditions (pH, ionic strengh, temperature), in presence of different inhibitors and after mutagenesis of residues involved in the active site, will be presented.

P 01

DIFFERENCES IN ACTIVE-SITE GORGE DIMENSIONS OF CHOLINESTERASES REVEALED BY BINDING OF INHIBITORS TO HUMAN BUTYRYLCHOLINESTERASE

Ashima Saxena[‡], Ann M.G.Redman[‡], Xuliang Jiang[§], Oksana Lockridge[#] and B.P. Doctor[‡]

[‡]Division of Biochemistry, Walter Reed Aimy Institute of Research, Washington, DC 20307, [§]The Integrated Program, College of Physicians and Surgeons of Columbia University, New York, NY 10032 and [#]Eppley Cancer Institute, University of Nebraska Medical Center, Omaha, NE 68198.

We examined the role of A328(*F330*) in the binding of various inhibitors to cholinesterases (ChEs) using human butyrylcholinesterase (BChE) mutants to determine if the conclusions drawn from studies with acetylcholinesterase (AChE) mutants could be extended to BChE. For huperzine A and edrophonium, the results obtained with AChE mutants could be directly correlated with those obtained with native ChEs and site-specific mutants of human BChE. Inhibition studies of ethopropazine with BChE mutants, where A328 was modified to either F or Y, suggested that A328 was not solely responsible for the selectivity of ethopropazine. Volume calculations for the active-site gorge showed that the poor inhibitory activity of ethopropazine towards AChE was due to the smaller dimension of the active-site gorge. The volume of the BChE active-site gorge is ~200 $Å^3$ larger than that of the AChE gorge which allows the accommodation of ethopropazine in two different orientations as demonstrated by rigid-body refinement and molecular dynamics calculations. These results suggest that although the overall scaffolding of the two enzymes may be highly similar, the dimensions and the micro-environment of the gorge play a significant role in determining the selectivity of substrate and inhibitors for ChEs.

P 02

Does Electrostatic Attraction or Steering by Charged Residues Within the Gorge Contribute to the Reactivity of AChE?

Arie Ordentlich[1], Dov Barak[2], Chanoh Kronman[1], Naomi Ariel[1], Baruch Velan[1] and Avigdor Shafferman[1]

Departments of [1]Biochemistry & Molecular Biology and [2]Organic Chemistry, Israel Institute for Biological Research, Ness-Ziona, 70450, Israel.

Studies with mutant AChEs where up to seven surface negative charges were neutralized demonstrated that electrostatic attraction does not contribute to the catalytic efficiency of the enzyme. Yet, electrostatic steering due to the negatively charged residues in the gorge (D74 at its rim and E202 and E450 near its bottom) remained difficult to assess. We therefore extended the studies of these mutants with isosteric pairs of charged and noncharged substrates (ATC- acetylthiocholine; 3,3-dimethylbutyl thioacetate-TB), charged and noncharged phosphate inhibitors (echothiophate, S-3,3-dimethylbutyl diethylthiophosphate) as well as with the transition state analog m-trimethylammonio trifluoroacetophenone (TFK). Replacements of D74 by negatively charged (D74E) positively charged (D74K) or neutral (D74N, D74G) residues resulted in small and uniform increase in values of Km for ATC except for D74K where the increase was much larger. On the other hand, the bimolecular rates of reactions with echothiophate and TFK were affected much more in cases of D74N and D74K than in those of D74E and E74G. All these mutations have almost no effect on catalytic activity toward the noncharged substrate, yet a small increase of phosphorylation rates by the noncharged OP inhibitor was observed for enzymes with noncharged residues at position 74 but not for D74K or D74E. These findings, together with the observation that ionic strength had small and equivalent effect on reactivities of all these enzymes, indicate that D74 probably does not enhance reactivity by steering charged ligands into the gorge but rather influences indirectly specific interactions of charged ligands with elements of the active center. Likewise, the reactivities of E202D, E202Q and E202A as well as that of E450A toward charged and noncharged ligands do not conform with the expected behavior due to electrostatic steering since substitutions of E202 have a major and similar effects on the values of kcat and phosphorylation constants for charged and noncharged substrates and OP inhibitors respectively. The results are consistent with the involvement of E202 in specific interactions, presumably with the catalytic triad residue H447, rather than in electrostatic steering.

P 03

ESMS as a Unique Tool for the Molecular Monitoring of Reactions Between HuAChE and Various OP-Agents

Arie Ordentlich[1], Ruth Barak[2], Dov Barak[3], Meir Fischer[4], Hendrik P. Benschop[5], Leo P.A. De Jong[5], Yoffi Segall[3], Baruch Velan[1] and Avigdor Shafferman[1]
[1]Department of Biochemistry and Molecular Genetics, [2]Department of Analytical Chemistry and [3]Department of Organic Chemistry, Israel Institute for Biological Research Ness-Ziona, 70450, Israel.
[4]Bio-Technology General (Israel) Ltd, Kiryat Weizmann Rehovot 76326 Israel
[5]Department of Chemical Toxicology, TNO Prins Maurits Laboratory, 2280 AA Rijswijk, The Netherlands.

The molecular masses of the bacterially expressed recombinant HuAChE and its conjugates with series of alkyl methylphosphonofluoridates and with diisopropyl fluorophosphate (DFP), were measured by electrospray - ionization mass spectrometry (ESMS). The mass of HuAChE (measured as 64700 Da, calc. 64695 Da) increased, following reactions with sarin, isobutyl methyl phosphorofluoridate (IBMPF), 1,2-dimethylpropyl methyl phosphorofluoridate (DMPF), soman and DFP, by 120, 140, 150, 160 and 160 Da respectively. These values were in excellent agreement with the calculated masses of the adducts and reflected both the addition of the phosphonyl moiety and the gradual mass increase due to branching of the alkoxy substituent. The composition of the phosphyl adducts change with time to yield a common product with molecular mass of 64780 Da, which is consistent with dealkylation of the phosphonyl moieties. By sequential ESMS measurements we were able to estimate the kinetics of evolution of the aged product of the HuAChE-soman adduct ($t_{1/2}$ = 50 sec, at pH 6.0). This rate is in good agreement with that determined by kinetic measurement of the development of non-reactivability under similar conditions. It is important to note that in agreement with the accepted mechanism of dealkylation, only two molecular species are evident in the sequential ESMS measurements during the aging process.

P 04

The Aromatic Moiety at Position-86 of HuAChE Accelerate the Aging of Phosphonyl-AChE Conjugates Through Cation-π Interactions

Dov Barak[1], Arie Ordentlich[2], Yoffi Segall[1], Baruch Velan[2], Hendrik P. Benschop[3], Leo P.A. De Jong[3] and Avigdor Shafferman[2]
Departments of [1]Organic Chemistry and [2]Biochemistry & Molecular Biology, Israel Institute for Biological Research, Ness-Ziona, 70450, Israel.
[3]Department of Chemical Toxicology, Prins Maurits Laboratory TNO, 2280 AA Rijswijk, The Netherlands.

The possible involvement of cation-π interactions in stabilization of partially charged transition states has been investigated through examination of the kinetics of dealkylation ('aging') for twelve alkyl methylphosphonyl conjugates of human acetylcholinesterase (HuAChE). These enzyme conjugates differ in the branching of the methylphosphono alkyl substituent and in the nature of the residue at position 86 of the enzyme. For conjugates of the wild type enzyme, a gradual decrease in the rates of aging, spanning two orders of magnitude, was observed for 1,2,2-trimethylpropyl; 1,2-dimethylpropyl; 2-butyl and 2-propyl methylphosphonyl moieties respectively. Substitution W86→F results in a moderate decrease in the rates of aging, irrespective of the nature of the phosphonyl moiety, suggesting a similar role of the aromatic residues in the aging process. The variation of aging rates due to branching of the alkyl substituent, for the W86A conjugates was within factor ten and resembled those of limiting solvolysis reactions. These findings demonstrate that the aromatic character of the residue at position 86 is an essential element of the enzymatic environment in facilitating the aging process, and that the extent of such involvement depends upon the branching of the alkyl methylphosphonyl moiety. The generality of the biocatalytic process including cation-π stabilization of carbocationic transition state, observed in aging of phosphylated AChEs, has recently gained support from other enzymatic systems.

P 05

ASSOCIATION OF TETRAMERS OF HUMAN BUTYRYLCHOLINESTERASE IS MEDIATED BY CONSERVED AROMATIC RESIDUES OF THE CARBOXY TERMINUS.

Cibby Varkey Altamirano and Oksana Lockridge
Department of Biochemistry and Molecular Biology, Eppley Institute, University of Nebraska Medical Center, 600 S. 42nd St., Omaha, NE 68198-6805, USA.

Human butyrylcholinesterase is composed predominantly of tetramers. Our laboratory has previously shown that up to 40 carboxy terminal residues of each subunit contribute to the stabilization of tetramers [Blong et al. (1997) *Biochem. J.* 327, 747-57]. To better define the residues which participate in tetramer stabilization, the *in vivo* interaction of the BChE C-terminus 46 residue peptide was quantitated for the wild type and mutant BChE using the yeast two-hybrid system. The wild type C-terminal peptides interacted with one another in this system. The C-terminal peptide mutants altering Cys 571 to Ala (C571A) or Ala 539 to Thr (A539T) interacted to a similar degree as the wild type peptides. However, only 11.7% of the interaction seen with the wild type peptide was observed with the mutant in which seven conserved aromatic residues (Trp 543, Phe 547, Trp 550, Tyr 553, Trp 557, Phe 561, and Tyr 564) had been altered to alanines (aromatics off mutant). To determine the effect of these C-terminal aromatic mutations on the oligomeric composition of BChE, the seven mutations were incorporated into the complete BChE molecule, with or without the C571A mutation and these mutants were expressed transiently in 293T cells. The aromatics off mutant contained no tetramers but was composed solely of dimers and monomers. In addition, the aromatics off mutant with the C571A mutation was composed of only monomers. Since Bon et al. [(1997) *JBC* 272: 3016-21] have observed that the addition of poly-L-proline to culture medium of cells expressing acetylcholinesterase increased the proportion of the tetrameric form, poly-L-proline was added to the media of transiently transfected 293T cells expressing wild type, G534stop (40 residues deleted from the C-terminus) or aromatics off BChE. For the wild type BChE, an increase in the tetrameric form and a corresponding decrease in the dimeric and monomeric forms were observed as the poly-L-proline concentration was increased. However, no increase in tetramers was observed with either the G534stop or the aromatics off BChE mutants. These observations suggest that the stabilization of BChE tetramers is mediated through the interaction of the seven conserved aromatic residues, Trp 543, Phe 547, Trp 550, Tyr 553, Trp 557, Phe 561, and Tyr 564, and that the poly-L-proline induced increase in tetrameric BChE is mediated through these seven aromatic residues.

Supported by U. S. Medical Research and Materiel Command DAMD 17-94-J-4005, DAMD17-97-1-7349, and AASERT award DAAG55-97-1-0244.

P 06

MOLECULAR MODELING OF TRANSITION STATES IN ACETYL-CHOLINESTERASE AND BUTYRYLCHOLINESTERASE INHIBITION BY CARBAMATES.

Zoran Radić
Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093-0636

Molecular models of carbamates bambuterol and RO-02-0683 were covalently attached to the active serine of mouse acetylcholinesterase (mAChE) and human butyrylcholinesterase (hBuChE) by creating a single bond between tetrahedral carbamyl carbon of the carbamate and Oγ of the serine, thus forming a putative adduct resembling the transition state.

Unlike RO-02-0683, bambuterol is a chiral molecule with one asymmetric carbon atom. Three starting conformations for each of two enantiomers of dicarbamate bambuterol in the transition state adduct were constructed by adjusting its dihedral angles to values calculated for the most stable conformers (Ekholm M. and Konschin H., *J.Mol.Struct.314(1994)277.*). Before running molecular dynamics, conformers were briefly minimized to relieve steric strain. The final conformation was obtained upon energy minimization. This procedure was repeated ten times for each enantiomer in each of the three starting conformations, in mAChE and in hBuChE. Six discrete families of final conformations resulted for both enzymes. The lowest energy conformers in mAChE and hBuChE differed but stereoselectivity was not observed in either enzyme. Differences in conformations were primarily evident in the choline binding site of two enzymes, and secondarily in the mAChE peripheral site, whereas no significant difference in the occupation of the acyl pockets was evident. While the leaving groups of the most stable conformers were pointing outside of the active center gorge in mAChE they resided in the choline binding site in hBuChE. The difference in orientation suggests that the tyrosine side chain in the choline binding site of mAChE is responsible, through steric constraints for the most of four orders of magnitude slower carbamylation of mAChE by bambuterol, as compared to hBuChE. The comparative analysis of the lowest energy conformers also suggests additional steric occlusion arising from the presence of Tyr 124 in the peripheral site of mAChE. Thus modeling results indicate that high specificity of bambuterol, for hBuChE compared to mAChE, depends on the difference in structure of the choline binding site, rather than the acyl pocket.

P 07

EPR LABELING OF ACETYLCHOLINESTERASE-FASCICULIN2 COMPLEX : MOLECULAR MODELS AND EXPERIMENT.

Zoran Radić[1], Marjeta Šentjurc[2], Slavko Pečar[3], Jure Stojan[4], and Zoran Grubič[5]

[1]Dept. of Pharmacology, Univ. of California at San Diego, La Jolla, CA (U.S.A.); [2]Jožef Stefan Institute, Ljubljana (Slovenia); [3]Faculty of Pharmacy, Ljubljana (Slovenia) and Institutes of [4]Biochemistry and [5]Patophysiology, School of Medicine, Ljubljana (Slovenia).

Molecular models of spin labeled organophosphate O-ethyl,O-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) fluorophosphate (EtOS1) covalently attached to the active serine (Ser203) of mouse AChE in complex with Fasciculin2 (FAS2) or the enzyme alone, were constructed by creating a single bond between phosphorus of the organophasphate and Oγ of the serine. The leaving group of the organophosphate was deleted.

EtOS1 is a chiral molecule with an asymmetric phosphorus. S- and R-enantiomers combined with AChE-FAS2 or AChE alone formed four different systems for the analysis. Before running molecular dynamics, conformers were initially minimized to relieve steric strain. The final conformation was obtained upon energy minimization. The procedure was repeated ten times for the each system. The resulting conformers of S-EtOS1 conjugates appeared more stable than R-EtOS1 ones in all systems. The presence of FAS2 bound to AChE induced slight shift of nitroxyl radical in both EtOS1 enantiomers toward the enzyme choline binding site. The S-EtOS1 radical approaches Trp86, and R-EtOS1 approaches Tyr337 to a distance of 3-4 A. At the same time Met33, the deepest protruding FAS2 residue into the enzyme active center gorge is 6-7 A away from the R-EtOS1 radical and about 10 A from the S-EtOS1 radical. Positioning of models of R- and in particular S-EtOS1 enantiomer in the gorge suggest that they would be able to sense conformational perturbations primarily in the choline binding site, induced by FAS2 binding. This suggestion was confirmed by analysis of EPR spectra of EtOS1 bound to AChE-FAS2 complex where differences in maximal hyperfine splitting were observed, indicative of restricted freedom of motion of the label upon FAS2 binding.

P 08

EFFECT OF HYDROSTATIC AND OSMOTIC PRESSURES ON THE RATE OF AGING OF DFP-PHOSPHORYLATED WILD-TYPE AND MUTANTS (E197D and D70G) OF BUTYRYLCHOLINESTERASE

C. Cléry, J. Clément, P. Guerra and P. Masson

Centre de Recherches du Service de Santé des Armées, Unité d'enzymologie, BP 87, 38702 La Tronche cedex, FRANCE

Organophosphates (pesticides, nerve agents) are potent inhibitors of cholinesterases (EC 3.1.1.7/8). The phosphylated enzymes can be reactivated by nucleophilic compounds such as oximes. With some organophosphates, the organophosphorus moiety of phosphylated enzymes undergoes a dealkylation, leading to non reactivatable, called "aged", enzymes.

In the present work we investigated the effect of hydrostatic pressure on the dealkylation of DFP-phosphorylated BuChE. Pressure dramatically increased the rate of "aging" of wt BuChE ($t_{1/2} \approx 1$ h at atmospheric pressure (P_0)), so that phosphorylated BuChE could not be reactivated by 2-PAM after pressure exposure, indicating that the activation volume $\Delta V^{\neq}$ for the dealkylation reaction is $<< 0$. Reactivation of the phosphorylated E197D ($t_{1/2} \approx 6$ h at P_0) and D70G ($t_{1/2} \approx 8$ h at P_0) mutants [1] was possible up to a pressure of 2.5 kbar for 15 h exposure at 25°C. For both mutants, the rate of aging under pressure slightly increased with hydrostatic pressure, giving $\Delta V^{\neq} \approx -3$ ml.mol.$^{-1}$. The effect of osmotic pressure provided information on the hydration changes in the "aging" process. Behaviour differences between phosphorylated wt and mutant enzymes under hydrostatic and osmotic pressures indicated that the mutations caused an extended change in the organization of the H-bond network in the active site gorge of BuChE having effects on the catalytic machinery of BuChE.

1-P. Masson, P. Fortier, C. Albaret, M. T. Froment, C. F. Bartels and O. Lockridge, Biochem. J., (1997) 325, 53-61.

The E197D and D70G BuChE mutants were provided by Dr. O. Lockridge (Omaha, NE, USA).

This research was supported by DSP grant N° DRET 96/12

P 09

GRAFTING OF GENETICALLY MODIFIED HUMAN FETAL FIBROBLAST TO PRODUCE HUMAN BUTYRYLCHOLINESTERASE IN MICE.

Haung SHAO, Yunzhong HAUNG, Danxin WANG, Han ZHANG, Manji SUN.
Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences; 307 Hospital, PLA. Beijing 100850, China.
E. mail: sunmj@anic.bmi.ac.cn

Human diploid fibroblast cultures were established from fetal skin tissue. Enzymatically dissociation yielded cultures of higher growth capacity of fibroblasts than those prepared by mechanical dissociation followed by spontaneous outgrowth of cells. Recombinant human butyrylcholinesterase (BChE, EC 3.1.1.8) gene transfering into primary human fibroblasts was achieved successfully using lipofection and retrovirus-mediated transfection. The analysis of drug-resistant colonies suggested the presence of the transcripted BChE mRNA in the cytoplasm of transfected cells. Secreted BChE protein in culture medium was assayed to be of enzyme activity using benzoylcholine and butyrylthiocholine as substrates. The fibroblasts genetically modified were mixed with rat tail collagen and transplanted subcutaneously and intraperitoneally to mice. Immunoreative human BChE appeared in the plasma from the transplanted mice sustained for about two weeks. Twenty days later, it did not present any longer in most of the mice.

P 10

Nonequilibrium analysis of acetylcholinesterase inhibition by propidium and gallamine

Tivadar Szegletes, William D. Mallender and Terrone L. Rosenberry

Department of Pharmacology, Mayo Foundation for Medical Education and Research, and the Department of Research, Mayo Clinic Jacksonville, Jacksonville, FL 32224, USA

A novel nonequilibrium approach to explain several features of the acetylcholinesterase active site was tested. The model was created to account for the inhibition of substrate hydrolysis observed when small ligands bind to the peripheral site at the mouth of the active site gorge. It proposes that this inhibition arises entirely by a steric blockade mechanism, i.e. a decrease in the association and dissociation rate constants for an acylation site ligand without altering the equilibrium constant for ligand binding to the acylation site. Furthermore, a decrease in the product dissociation rate by bound peripheral site inhibitors was also incorporated into the model. Simulations based on this nonequilibrium steric blockade model were in good agreement with experimental data. Simulated inhibition by propidium and gallamine of either acetylthiocholine or phenyl acetate hydrolysis agreed with measured values when binding of these inhibitors to the peripheral site slowed substrate association and dissociation rate constants (5- to 70-fold). Direct measurements with the acylation site ligands huperzine A and *m-(N,N,N*-trimethylammonio)trifluoro-acetophenone showed that bound propidium decreased both the association rate constants (49- and 380-fold) and the dissociation rate constants (10- and 60-fold), relative to the rate constants for these acylation site ligands with free AChE. We conclude that this model can account for the inhibition of AChE by small peripheral site ligands and for substrate inhibition without invoking any conformational interaction between the peripheral and acylation sites.

P 11

ROLE OF EDROPHONIUM IN PREVENTION OF THE RE-INHIBITION OF ACETYLCHOLINESTERASE BY PHOSPHORYLATED OXIME

Chunyuan Luo[1], Ashima Saxena[1], Zoran Radić[2], Yacov Ashani[3], Palmer Taylor[2]and Bhupendra P. Doctor[1]
[1]Walter Reed Army Institute of Research, Washington,DC 20307-5100,
[2]niversity of California at San Diego, California 92093-0636 and
[3]Israel Institute for Biological Research, Ness-Ziona, Israel

We further examined the role of edrophonium on acceleration of oximeinduced reactivation of acetycholinesterase (AChE) inhibited by 7-(O,O-diethylphosphinyloxy)-1-methylquinulinium methylsulfate (DEPQ). The results indicated again that the edrophonium-induced acceleration is due to the formation of a reversible complex between AChE and edrophonium, which prevents the re-inhibition of the reactivated enzyme by the putative phosphorylated oxime (POX). Among all the oximes tested, LüH6 and TMB4 were found to be the most significant in edrophonium-induced acceleration. In experiments with mouse AChE mutant D74N, for which the inhibition potency of charged OP decreased 2-3 orders than wild-type enzyme, the acceleration effect of edrophonium disappeared, and the time courses of reactivation showed a typical 1st order kinetic process with both oximes LüH6 and TMB4. The data from mutant enzyme substantiate the involvement of POX re-inhibition in the edrophonium acceleration on oxime-induced reactivation. In addition, in the presence of rabbit serum OP hydrolase, significant acceleration of oxime-induced reactivation of DEPQ-inhibited AChE was also observed with LüH6 and TMB4. The acceleration by OP hydrolase and edrophonium seemed to be additive, indicating the rabbit serum OP hydrolase could hydrolyze the POX formed during the reactivation process.

P 12

ACCELERATION OF OXIME-INDUCED REACTIVATION OF MEPQ-INHIBITED ACETYLCHOLINESTERASE BY MONO- AND BIS-QUATERNARY LIGANDS

Chunyuan Luo,[1] Yacov Ashani,[2] and Bhupendra P. Doctor[1]
[1]Walter Reed Army Institute of Research, Washington, DC 20307-5100 and [2]Israel Institute for Biological Research, Ness-Ziona, Israel

We examined ligand modulations of oxime-induced reactivation of methylphosphonylated AChE using 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ) and fetal bovine serum AChE. Edro-phonium, decamethonium, and propidium, three quaternary AChE ligands of different types, were tested as potential accelerators. Kinetic measurements with oximes 2-PAM, TMB_4, and $L\ddot{u}H_6$ showed that in the presence of 50 μM edrophonium the reactivation rate constants increased 3.3 to 12.0-fold; 200 μM decamethonium produced 1.6 to 3.0-fold enhancement of reactivation rate constants by the same oximes. Reactivation of the MEPQ-inhibited enzyme by HI-6, HS-6, and HLo7 was not affected by either ligand. Propidium slowed the reactivation of MEPQ-inhibited AChE by all oximes. Results suggest that accelerator site may reside inside the catalytic gorge rather than at its entrance, and acceleration may be due to the prevention of re-inhibition of regenerated enzyme by the putative product, the phosphonylated oxime (POX). In addition to the nucleophilic property of the oximate anion, some of the reactivators may carry an accelerating determinant, as characterized with respect to edrophonium and decamethonium. Results offer possible ex-planation for the superiority of HI-6 over other oximes in reactivation of specific AChE-OP conjugates.

P 13

AMINO ACIDS INVOLVED IN THE INHIBITION OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE BY Ro 02-0683 AND BAMBUTEROL

Z. Kovarik, M. Škrinjarić-Špoljar, B. Grgas, *Z. Radić and V. Simeon-Rudolf

*Institute for Medical Research and Occupational Health, 10001 Zagreb, Croatia, *Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093-0636, USA*

In order to identify amino acids involved in interaction of acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) with Ro 02-0683 (dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethyl-ammonium bromide) and bambuterol (5-[-(*tert*-butylamino)-1-hydroxyethyl]-m-phenylene-bis (dimethylcarbamate) hydrochloride) the time course of inhibition by these two carbamates was studied. Recombinant mouse wild-type AChE and BChE, site-directed AchE mutants and native human serum BChE phenotypes were inhibited by varying concentrations of the carbamates. Both inhibitors are charged compounds; Ro 02-0683 has a quaternary amino group while the secondary amine (pK_a = 9.61) of bambuterol is almost completely quaternized at our experimental conditions (0.1 M phosphate buffer, pH = 7.4, 25 °C).

The second order rate constants of inhibition of mouse BChE and human serum BChE usual and fluoride-resistant variants were similar for both carbamates. The rate constant of inhibition of AChE by Ro 02-0683 was on average 10-times and that by bambuterol 16000-times smaller than that of BChE. Aspartat^{74}asparagine (D74N) AChE mutant was inhibited slower by both inhibitors than the wild-type AChE. Likewise, the inhibition of atypical human BChE (Asp^{70}Gly natural mutation) by both inhibitors was slower than that of the usual enzyme. Both carbamates were better inhibitors of the peripheral site-directed AChE mutant, tyrosine^{124}glutamine (Y124Q), than of the wild-type AChE. Bambuterol was a better and Ro 02-0683 was a worse inhibitor of the choline binding site mutant tyrosine^{337}alanin (Y337A) than of the wild-type AChE. The latter two amino acid substitutions mimic elements of BChE structure critical for its specificity on the AChE template.

P 14

KINETICS OF INHIBITION OF HUMAN SERUM BUTYRYLCHOLINESTERASE (EC 3.1.1.8) HETEROZYGOUS PHENOTYPES BY THE DIMETHYLCARMABATE Ro 02-0683

V. Simeon-Rudolf, Z. Kovarik, A. Buntić, M. Škrinjarić-Špoljar and *R. T. Evans

*Institute for Medical Research and Occupational Health, P.O.Box 291 10001 Zagreb, Croatia,*Leeds, UK*

The time course of inhibition by the dimethylcarbamate Ro 02-0683 of sera heterozygous for butyrylcholinesterase phenotyped by the inhibitor numbers as UA, UF, AF, AK or AJ, was followed from zero to 120 min by measuring the remaining activity using propionylthiocholine as substrate. The aim was to determine the rate constants of inhibition of heterozygous enzyme and of each variant as well as its contribution to the total activity. The reaction does not follow first order kinetics and it can be explained by the presence of two enzyme variants in heterozygous sera which are inhibited to different extents. The remaining activities (y / %) vs. time (t) were fitted to the bi-exponential equation $y = Q\ exp\ (-k_Q\ t) + Z\ exp\ (-k_Z\ t)$ where Q and Z are activity contributions of the U, F, A, K or J variants to the total activity of the heterozygous serum and k_Q and k_Z the respective first order rate constants of inhibition. The time course of inhibition for sera of UU, UK and UJ phenotypes is similar. This substantiates the earlier findings that K and J mutations lead to quantitative changes in the concentration of usual enzyme in contrast to the qualitative changes of the atypical and fluoride resistant variants. The second order rate constants of inhibition by Ro 02-0683 calculated from the time course of inhibition of UA, AK, AJ, UF and AF sera were 9.0, 10, 11, and 3.9 L $\mu mol^{-1} min^{-1}$ for U, K, J and F variants respectively. Contributions of the atypical variant to the total activity of UA, AK, AJ and AF heterozygous sera were 18, 28, 43 and 41% respectively. The difference between the Ro 02-0683 inhibitor numbers for UA, AK and AJ heterozygots is the result of the different contribution of U, K or J variant to the total activity of atypical heterozygots.

This study was part of an ALIS project supported by the British Council and the Ministry of Science and Technology of the Republic of Croatia.

P 15

CATALYTIC PARAMETERS FOR THE HYDROLYSIS OF BUTYRYLTHIOCHOLINE BY HUMAN SERUM BUTYRYLCHOLINESTERASE PHENOTYPES

V. Simeon-Rudolf, E. Reiner, *R. T. Evans, **P. George and **H. Potter

*Institute for Medical Research and Occupational Health, P.O.Box 291 10001 Zagreb, Croatia, *Leeds, UK, **Molecular Pathology Group, Christchurch Hospital, Christchurch, New Zealand*

The hydrolysis of butyrylthiocholine (BTCh) by the usual (UU), fluoride-resistant (FS), AK, AJ and atypical (AA) human serum butyrylcholinesterase (EC 3.1.1.8) phenotypes was measured in phosphate buffer pH=7.4 at 25 °C. Three sera were used for each phenotype taken from individuals whose cholinesterase phenotype had previously been determined by measurement of the activities and inhibition by dibucain, sodium fluoride and the dimethylcarbamate Ro 02-0683. pS curves for all phenotypes were S-shaped; the activities rose to a plateau with increasing substrate concentration except at 100 mM where there was a small decrease. To calculated the catalytic constants three equations were applied: Michaelis-Menten (1), Hill (2) and an equation which assumes simultaneous binding of the substrate to the catalytic site and to a peripheral site on the enzyme (3). Over the range from 0.01 to 100 mM BTCh the activity vs. substrate concentration relationship deviated from Michaelis-Menten kinetics while the pS-curves fitted well with eqns. 2 and 3. The Michaelis-Menten equation was applied separately to two BTCh concentration ranges. The Km constants for the five phenotypes ranged from 0.1 to 0.2 mM (at 0.01 - 1.0 mM BTCh) and from 0.4 to 2.0 mM (at 1.0 - 50 mM BTCh). Hill coefficients (n) calculated from eqn.2 were similar ($n \cong 0.6$) for all phenotypes. The binding constants K_1 and K_2 calculated from eqn. 3 for two sites on the enzyme fell between 0.02 and 0.12 mM (K_1) and 0.89 and 4.9 mM (K_2). The Vm values calculated from all three equations agreed with maximal activities obtained experimentally. Application of the three equations to our experimental data supports the argument that butyrylcholinesterase has two substrate binding sites.

This study was part of an ALIS project supported by the British Council and the Ministry of Science and Technology of the Republic of Croatia.

P 16

TRYPTOPHAN RESIDUE(S) AS MAJOR COMPONENT(S) OF THE HUMAN PARAOXONASE ACTIVE SITE.

D. Josse[a,b], Wei Hua Xie[a], P. Masson[b], L.M. Schopfer and O. Lockridge[a]
[a]Eppley Institute, University of Nebraska Medical Center, Omaha, NE 68198-6805, USA and [b]Unite d'enzymologie, CRSSA, La Tronche 38702, FRANCE

Paraoxonase (PON) is an HDL-associated, calcium-dependent enzyme whose 3D structure, active site residues and physiological substrates are not known. The kinetic parameters k_{cat} and Km (relative to k_{cat} and Km of the wild-type), determined with four substrates (phenylacetate, paraoxon, diazoxon and chlorpyrifos oxon) were less than 1% and 100% for the W280A and W280F mutant enzymes, respectively. These results indicated that the aromatic/hydrophobic character of the amino acid in position 280 is essential for PON activity.

In this study, we investigated whether this aromatic residue is in the PON active site. Group-specific labelling studies with N-bromosuccinimide, an oxidative agent of tryptophan, strongly suggested that one or several Trp could be in the active site of PON but we could not conclude either on the specificity of the labelling reaction or on the number of oxidized Trp. However, although PON activity was not altered by the hydrophilic tryptophan-modifying reagent 2-hydroxy-5-nitrobenzyl chloride (NBC), it was significantly reduced by the p-nitrophenylacetate analog 2-acetoxy-5-nitrobenzyl chloride (ANBC), whose hydrolysis by PON generated NBC in the active site. Moreover, since at least one calcium ion is present in the PON catalytic site, we attempted to probe the metal local environment using the calcium analog terbium. The luminescence spectrum of the PON-terbium complex exhibited an emission peak at 545 nm characteristic of an aromatic residue (Trp and/or Tyr)-terbium interaction.

In conclusion, both the results obtained with the mechanism-based inhibitor of PON (ANBC) and the calcium binding site luminescent probe terbium support the hypothesis of the presence of at least one Trp residue in the PON active site. Trp residue(s) may be involved in the binding of aromatic substrates.

Supported by DCSSA to D.J. and P.M. and U.S.Med.Res.& Mat. Command DAMD 17-94-5-4005, DAMD 17-97-1-7349 to O.L.

P 17

HUMAN SERUM PARAOXONASE : IDENTIFICATION OF ESSENTIAL AMINO ACID RESIDUES BY GROUP-SPECIFIC LABELLING AND SITE DIRECTED MUTAGENESIS.

D. Josse[a,b], Wei Hua Xie[a], P. Masson[b] and O. Lockridge[a]
[a]Eppley Institute, University of Nebraska Medical Center, Omaha, NE 68198-6805, USA and [b]Unite d'enzymologie, CRSSA, La Tronche 38702, FRANCE.

Human serum paraoxonase/arylesterase (PON, E.C.3.1.8.1) is a calcium-dependent enzyme which hydrolyzes a wide variety of organophosphates, including paraoxon, DFP, sarin and soman. Although the 3D structure of PON has not yet been determined and its sequence shows no similarity with any other proteins, we undertook to identify some of its essential amino acid residues by two complementary approaches : group-specific labelling and site-directed mutagenesis. Group-specific labelling studies, performed on the purified native enzyme, indicated that one or more Trp, His and Asp/Glu are potentially important residues for PON activity. Based on these results, we identified some of these residues, conserved in the sequenced mammalian PON, by site-directed mutagenesis. PON mutants were transiently expressed in 293T cells. The catalytic constants k_{cat} and Km (relative to k_{cat} and Km of the wild-type) determined with four different substrates (phenylacetate, paraoxon, diazoxon, chlorpyrifos oxon), were not significantly changed for the following mutants : W193A, W201A, W253A, H160N, H245N, H250N, H347N, E32A, E48A, D88A, D107A, D121A, D273A. By contrast, k_{cat} was less than 1% for eight mutants : W280A, H114N, H133N, H154N, H242N, H284N, E52A and D53A. The essential amino acid residues identified in this work could be part of the PON active site, acting either as calcium ligands (E52 and D53?) or as substrate binding (W280?) or nucleophilic (His residues?) sites. However, we cannot rule out that the effects of mutations on catalytic properties resulted from a remote conformational change and/or misfolding mutant proteins.
Supported by DCSSA to D.J. and P.M. and U.S.Med.Res.& Mat. Command DAMD 17-94-5-4005, DAMD 17-97-1-7349 to O.L.

P 18

ACETYLCHOLINESTERASE KNOCKOUT MOUSE.

Wei Hua Xie (1), P.J.Wilder (1), J.Stribley (2), A. Chatonnet (3), A. Rizzino (1), P. Taylor (4), S. Hinrichs (2) and O. Lockridge (1).

(1) Eppley Institute, Univ. Nebraska Med. Ctr., 600 S. 42nd St., Omaha, NE 68198-6805. (2) Path. Micro. Dept., Univ. Nebraska Med. Ctr. (3) INRA, Montpellier, France. (4) Pharmacology Dept., Univ. California San Diego

The goal of this work is to determine whether AChE is essential for normal development of the mouse embryo. One allele of the ACHE gene was knocked out of ES cells by homologous recombination. The targeting vector contained 2 kb of a TK cassette for negative selection, 884 bp of ACHE including exon 1, 1.6 kb of a Neo^r gene cassette for positive selection, 5.2 kb of the ACHE Bam HI fragment including exon 6, and 3 kb of Bluescript. Deletion of exons 2-5 removed 80% of the ACHE coding sequence. The gene targeting vector was introduced into embryonic stem cells by electroporation. Colonies resistant to G418 and gancyclovir were screened for homologous recombination by Southern blotting. Out of 200 colonies, 4 were found to have undergone homologous recombination. These 4 ES cell lines were expanded to provide cells for injection into C57BL/6 mouse blastocysts. The injected blastocysts were implanted into pseudopregnant white mice. More than 200 injected blastocysts were transferred into 20 mice. A total of 65 mice were born of which 5 were chimeras. Chimeras were identified by their black and brown (agouti) coat color. Littermates were all black. Four male chimeras are being bred with C57BL/6 females to determine if the knockout is in the germline. Offspring with agouti coat color have a 50% chance of carrying the ACHE knockout. We anticipate a phenotype will appear in the perinatal period of heterozygous animals or in utero in homozygous animals. By the time of the meeting we expect to know whether mice heterozygous for the knockout survive to adulthood.

Supported by U.S.Army Medical Research and Materiel Command DAMD17-94-J-4005, DAMD17-97-1-7349, Association Francaise Contre les Myopathies (MNM1997), and Nebraska State Research Initiative.

P 19

EVIDENCE THAT SEVERAL CONSERVED HISTIDINE RESIDUES ARE REQUIRED FOR HYDROLYTIC ACTIVITY OF HUMAN PARAOXONASE/ARYLESTERASE

J.A. Doorn, R.C. Sorenson, S.S. Billecke, C. Hsu, B.N. La Du.
Dept. of Environmental and Industrial Health, School of Public Health. Depts. of Anesthesiology/Pharmacology, Medical School. University of Michigan, Ann Arbor, MI, 48109, U.S.A.

Recent evidence has been acquired that implicates an important role for several histidine residues in the hydrolytic mechanism of human paraoxonase/arylesterase (PON1). Following titration with diethylpyrocarbonate (DEPC) both human serum and recombinant human type Q PON1 were inhibited in respect to their hydrolytic activity in a dose-responsive manner. DEPC contains two electrophilic carbon atoms which are reactive with the imidazole side chain of histidine residues (Miles, *Methods Enzymol.* **47**, 431-442,1977). The reaction yielded N-carbethoxy-hystidyl derivatives, and the formation of product was followed spectrophotometrically by the increase in absorbance at 244 nm. Subsequent treatment with hydroxylamine removed the N-carbethoxy group and partially restored enzymatic activity. Human PON1 treated with varying concentrations of DEPC lost hydrolytic activity, and with each histidine modified there was an exponential drop in hydrolytic activity.

Recombinant wild-type and C283A PON1 enzymes inhibited with DEPC and subsequently treated with hydroxylamine had partial restoration of hydrolytic activity. The dose response and time of DEPC inactivation was similar for both of these enzymes. The C283A mutant lacks a free sulfhydryl group, indicating that its inactivation is due to histidine specific modification. The extent of restoration of hydrolytic activity was similar for both wild-type and C283A recombinant enzymes which also indicates that DEPC inactivation is the result of histidine modification since hydroxylamine treatment cannot remove an S-carbethoxy group (Melchior and Fahrnney, *Biochemistry* **9**, 251, 1970).

Mutants of PON1 containing alanine or arginine residues substituted for each of several conserved histidine residues lost hydrolytic activity for each single substitution. The mutants of PON1 constructed and assayed for arylesterase activity were H114A, H114N, H133N, H154N, H284A, and H284N. Each single amino acid substitution rendered the enzyme catalytically inactive.

These two pieces of evidence implicate an important role for several histidine residues in the hydrolytic mechanism of PON1. Although it is unusual for a calcium dependent enzyme to require histidines for its catalytic activity, acquired data suggest such a circumstance.

P 20

SERUM ALBUMIN CAN HYDROLYSE ORGANOPHOSPHORUS COMPOUNDS BY AN INTERMEDIATE PHOSPHORYLATED MECHANISM. Where is the borderline dividing A and B-esterases?

M.A. Sogorb, A. Monroy, J.L.Vicedo, J. Barril, E. Vilanova
Universidad Miguel Hernandez. Unit of Toxicology and Chemical Safety. Campus de San Juan (Alicante). Spain.

We have demonstrated that serum albumins contain activities hydrolysing dichlorophenyl phosphoramidates (HDCPase) in a non-calcium dependent manner. Albumin also shows CbE activity when tested with p-nitrophenylbutirate (pNPB). Both activities (HDCPase and pNPBase) seem to be associated to the same active centre as competitive inhibition between both substrates have been demonstrated.

The time course of the reaction shows an initial fast phase followed by a slow phase. The hypothesis of a mechanism based on an intermediate phosphorylated stage has been tested and dissected by experiments based in the interaction between both substrates. Washing out the substrate (by dilution or ultrafiltration) after finish the initial fast phase the activity remains with low rate (partly inhibited) for some time when assayed with the same or the other substrate. Activity was recovered to the level of the initial fast rate after longer time in a way that is quantitatively compatible with the hypothesis that the rate of recovery by dephosphorylation (with the OP substrate) or deacylation (with the carboxylester substrate) accounts for the rate of the slow phase.

The term A-esterases is used for the carboxylesterases (CbE) able to hydrolyse OPs, and B-esterase to those CbEs that are inhibited (phosphorylated) by OPs. We concluded that albumins can hydrolyse OPs by a mechanism of the type of a B-esterase with a covalent acetylated (phosphorylated) intermediate. We suggest differentiating them by calling as **AB-esterases**, being actually and mechanistically a B-esterase but with dephosphorylation rate to functionally catalyse the OP hydrolysis.

Work supported by grant 5AF96/0168 (CYCIT, Spain) and FIS95/0053 (FIS, Spain).

P 21

PROPERTIES OF THE RETAINED N-TERMINAL HYDROPHOBIC LEADER SEQUENCE IN HUMAN SERUM PARAOXONASE/ARYLESTERASE

Robert C. Sorenson[1], Michael Aviram[2], Charles L. Bisgaier[3], Scott Billecke[1], Cary Hsu[1], and Bert N. La Du[1]

[1]Departments of Pharmacology and Anesthesiology, University of Michigan Medical School, Ann Arbor, MI 48109; [2]Lipid Research Laboratory, Rambam Medical Center and the Bruce Rappaport Faculty of Medicine, Technion and the Rappaport Family Institute for Research in the Medscal Sciences, Haifa Israel 31096: [3]Parke-Davis/Warner-Lambert, Ann Arbor, MI

Human serum paraoxonase/arylesterase (PON1) is HDL-associated and may protect low density lipoproteins (LDL) from oxidation. Mature PON1 retains its N-terminal signal sequence, which may be needed for binding to HDL. The peptide may be retained because of large, polar residues at sites where smaller, neutral residues are usually found at the cleavage site. By site-directed mutagenesis, we created a cleavable N-terminus by replacing residues 19H and 20Q with alanines. PON1(type Q)A19A20 is secreted, glycosylated, retains immunoreactivity with anti-PON1 anti-sera, shows a reduction in molecular weight consistent with N-terminal cleavage, and retains arylesterase and paraoxonase activities (although greatly reduced). These data indicate that the mutant protein is processed, and secreted similarly to the wild type protein. The wild type recombinant enzyme binds to apoA1-free phospholipid vesicles as well as apoA1-containing vesicles in size-exclusion chromatography, whereas the A19A20 mutant binds neither, demonstrating that the retained N-terminal peptide is necessary for phospholipid binding. It is not yet clear whether this peptide interacts with apoA1. To assess the role of the retained N-terminal sequence for PON1's protection against LDL oxidation, we used a copper ion-induced oxidation system and found that the wild type enzyme and A19A20 mutant showed similar reductions in both peroxide and aldehyde formation, up to 40%. We conclude that changing residues H19 and Q20 to alanines results in cleavage of PON1's N-terminal signal sequence and reduces hydrolytic activity. It is also clear that retention of the N-terminal sequence in PON1 is necessary for phospholipid binding but is not needed for processing, secretion, or many of PON1's activities.

P 22

IMMUNOLOCALIZATION OF PARAOXONASE IN RAT TISSUES

Antonio F. Hernández, Jose J. López, Lourdes Rodrigo, Fernando Gil, and Antonio Pla.

Department of Legal Medicine and Toxicology Service. Faculty of Medicine. University of Granada. Spain.

Studies on the localization of paraoxonase are of interest because of its involvement in both the detoxication of activated organophosphorus pesticides (of which parathion is a typical example) and in the prevention of peroxidative damage to phospholipids in LDL particles and cells membranes along the atherogenic process. In this study, the distribution of paraoxonase protein in rat tissues has been investigated by immunohistochemistry, using an antibody raised against a highly purified rat liver paraoxonase. Paraoxonase antigen was mainly detected in hepatocytes from the centrolobular region and within endothelial cells in rat liver sections. Bile ducts lacked immunostaining; however, plasma trapped within vascular spaces showed crossed-immunorreactivity, what suggests that serum paraoxonase can also be detected using antibodies against liver microsomal paraoxonase. Similarly, we have investigated the distribution and localization of paraoxonase by immunohistochemistry in a range of rat tissues from either 3-methylcholantrene induced-rats or the corresponding normal tissues. Paraoxonase was preferently detected in the endothelial lining of every tissue studied (lung, kidney and brain). The immunostaining pattern showed a clear functional polarisation in each tissue as follows: *(a)* Lung: paraoxonase was localised in cells from bronchiolar epitellium as well as in Type I pneumocytes; *(b)* Kidney: glomerulus and vascular side of proximal convoluted tubule; *(c)* Brain: leptomeningeal cells, ependimary cells and ventricular side of choroid plexus cells. After induction protocol with 40 mg/kg of 3-methylcholantrene, an increased immunostaining was observed in every tissue studied, as well as paraoxonase overexpression in liver, especially in hepatocytes other than those from centrolobular region, with tendency to be localised in the vascular side of the cells.

P 23

IDENTIFICATION OF TWO RAT LIVER PROTEINS WITH PARAOXONASE ACTIVITY

Lourdes Rodrigo, Fernando Gil, Antonio F. Hernández and Antonio Pla
Department of Legal Medicine and Toxicology Service, University of Granada, Avda. Madrid, 11. E-18071 Granada, Spain.

The existence of two or more enzyme forms with paraoxonase activity has been reported in sheep, rabbit, human and rat serum. In this study we describe the presence of two peaks with paraoxonase activity (A and B) after non-specific affinity chromatography of rat liver microsomes on Cibacron Blue 3GA. Sample was applied on a column of Cibacron Blue after solubilization of microsomal fraction, hydroxyapatite adsorption and chromatography on DEAE-Sepharose CL-6B. Two peaks of paraoxonase activity were detected, in contrast to previous chromatographic steps where only a single peak with paraoxonase activity was identified. The first peak (A) with paraoxonase activity was obtained during the washing of the column and coeluted with albumin. The second active peak (B) was eluted with 1M ClNa. The activity of peak A was 20-25% of the total activity recovered in this chromatographic step. SDS-PAGE analysis of these two active peaks showed a similar molecular mass (45000) for both enzyme activities. Furthermore, the band with paraoxonase activity associated with peak A was clearly separated from the albumin. A comparison of both active fractions (A and B) has been performed on the basis of kinetic parameters, heat inactivation and pH stability, calcium requirement, and inhibition by EDTA and several metals.

P 24

FURTHER EVIDENCE FOR THE IDENTITY OF PARAOXONASE AND ARYLESTERASE ACTIVITIES IN RAT LIVER

Lourdes Rodrigo, Fernando Gil, Antonio F. Hernández, and Antonio Pla
Department of Legal Medicine and Toxicology Service, University of Granada, Avda. Madrid, 11. E-18071 Granada, Spain.

Hydrolysis of several organophosphorus compounds has been usually associated to arylesterase (ArE) activity (E.C. 3.1.1.2). According to recommendations of different authors, the IUB (1992) added a new heading for the Phosphoric triester hydrolases (E.C. 3.1.8) considering arylesterase (E.C. 3.1.1.2) and paraoxonase (E.C. 3.1.8.1) as different enzymes. Nevertheless, genetic evidence that the same enzyme is capable to hydrolyze both paraoxon and phenylacetate have been reported in the last years. Our results support, under a biochemical point of view, the identity between paraoxonase and arylesterase activities. During the purification to homogenity of rat liver paraoxonase we have performed a study of its hydrolytic ability against three different substrates: paraoxon (Pxnase), phenylacetate (ArE) and phenylthioacetate (PTase) using as identity criteria for the three hydrolytic activities the elution profile in different chromatographic steps as well as the activity ratios from the crude extract throughout the purification process. Our results show an identical chromatographic behaviour, suggesting that the three hydrolytic activities are expressed by the same enzyme protein. Every fraction with paraoxonase activity was also capable to hydrolyze the other two substrates, lacking evidence for the existence of different enzymes for the hydrolysis of paraoxon and phenylacetate. The same conclusion can be drawn from the analysis of activity ratios Pxnase/ArE and ArE/PTase.

P 25

PERIPHERAL NERVE SOLUBLE ESTERASES ARE SPONTANEOUSLY REACTIVATED AFTER INHIBITION BY SOME OP COMPOUNDS: Implications for a new definition of NTE

*Barril, J. *, Estévez, .J, Escudero, M.A., Céspedes, M. V., Ńiguez, N., Sogorb, M.A., Monroy, A., and Vilanova, E.* Universidad Miguel Hernández. Laboratorio de Neurotoxicologia. Alicante. Spain.

Paraoxon, a non-neuropathic organophosphorus compound (OP) has been shown to strongly interfere with the mipafox inhibition of peripheral nerve soluble neuropathy target esterase (S-NTE) (1). Removal of paraoxon (by ultrafiltration) in order to avoid such an interference results in the reactivation of up to 80% of the total soluble phenyl valerate esterase (PVase) activity apparently inhibited by paraoxon (reversibly?). Mipafox, a neuropathic OP, inhibits more than 90% of PVases in tissue pretrated with paraoxon and removed out ("PX"-tissue). However, one third of them are reactivated on washing out of the mipafox. A reversible inhibition might be suspected. However, the kinetics study showed that both OPs inhibit the Pvases in a time-progressive manner leading to the conclusion that they have an unusually high reactivation constant for their interaction with some of these soluble PVases. Gel filtration chromatography of "PX" tissue in Sephacryl S-300 show that only PVases associated with S-NTE2 (the 100 kD peak) (2, 3) are reactivated. The implications of these findings in the definition of NTE in a target tissue for the so-called organophosphorus induced delayed polyneuropathy (OPIDP) are discussed.

Work partly supported by FIS 95/0053. (1) Barril, J. and Vilanova E. (1997). Chem.-Biol. Interact. (in press). (2) Escudero, M.A., Barril, J., Tormo, N. and Vilanova E. (1995). Chem.-Biol. Interact. 97, 247-255. (3) Escudero, M.A. and Vilanova E. (1997). J. Neurochem. 69,1975-1982.

P 26

CHICKEN LIVER PHENYL VALERATE ESTERASES DISCRIMINATED BY PARAOXON AND MIPAFOX INHIBITION.

M.A. Escudero, J. Barril, M-V. Céspedes, E. Vilanova. Universidad "Miguel Hernández". Campus de San Juan (Alicante). Spain.

Chicken liver homogenate shows highest level of phenylvalerate esterases (PVases) in all studied tissues: 90,300 nmol/min//g fresh tissue. The relative content of particulate/soluble activity was 45/55 % respectively. Using paraoxon and mipafox in ususual concurrent inhibition procedure, the level of operationally NTE (40 uM paraoxon-resistant, 250 uM mipafox-sensitive PVase) was 1.8% of total PVase: 1,587±197 nmol/min/g in homogenate, being 34% in soluble form therefore the liver has similar "NTE" content than brain. The paraoxon resistant and NTE activity was much higher when paraoxon was removed out before mipafox inhibition than when used concurrently, suggesting that the activity inhibited by paraoxon is reversibly reactivated when removed out. Contradictory, the paraoxon inhibited most PVase in time progressive manner, suggesting covalent inhibition. Most of the paraoxon resistant (after removing), is sensitive to mipafox in a time progressive manner with a inhibition Ka of 0.098 and 0.54 min^{-1} uM^{-1}, in particle and soluble fraction, respectively. The I50 to mipafox deduced from the Ka (for 30 min) was about 240 and 34 nM in particles/soluble, respectively.
Neuropathy target esterase (NTE) is operat. Work partly supported by grants FIS-95.0053

P 27

A NEW WAY FOR DETERMINATION OF NEUROPATHY TARGET ESTERASE ACTIVITY

[1)]L.V. Sigolaeva*, [2)]A.V. Eremenko, [3)]G.F. Makhaeva, [3)]V.V. Malygin, [1,2)]I.N. Kurochkin

[1)]Faculty of Chemistry, M. V. Lomonosov State University, Moscow, 119899 Russia, [2)]Research Center for Molecular Diagnostics and Therapy, Moscow, 113149 Russia, [3)]Institute of Physiologically Active Substances, Chernogolovka, 142432 Russia

Some of OPs were found to induce a delayed neuropathy (OPIDN). A specific target for OPIDN initiation is neuronal protein known as Neuropathy Target Esterase (neurotoxic esterase, NTE). The correlation is occur between the ability of OP to inhibit NTE activity and to induce OPIDN.

A biosensor for the NTE activity measurements was constructed based on a Clark-type oxygen electrode modifed by immobilized tyrosinase. The analytical signal of amperometric biosensor (oxygen consumption detected by Clark electrode) is proportional to the quantity of phenol. The latter is the product of enzymatic hydrolysis of phenyl valerate by NTE. At the same time phenol is the substrate for the enzymatic oxidation to catechol and finally to 1,2benzoquinone by tyrosinase proceeding with oxygen consumption. The experiments were carried out for realizing a chemically amplified signal by combining the tyrosinase reaction and a reducing agent (L-ascorbic acid) in order to decrease the level of tyrosinase based biosensor sensitivity.

The analytical response of biosensor is linear in the range of phenol concentrations from $5x10^{-8}$ M to $1x10^{-6}$ M. Minimum concentration of NTE that can be determined in standard conditions by this biosensor is approximately $2x10^{-9}$ M.

It is expected that this sensitive, simple and low-cost biosensor's method can be used either for the environmental monitoring of organophosphorus delayed neurotoxicants and for early clinical diagnostics of OPIDN.

P 28

A STABLE PREPARATION OF HEN BRAIN NEUROPATHY TARGET ESTERASE FOR RAPID BIOCHEMICAL ASSESSMENT OF NEUROTOXIC POTENTIAL OF ORGANOPHOSPHATES (OP)

G.Makhaeva, V.Malygin, Institute of Physiologically Active Substances Russian Academy of Science, Chernogolovka,142432, RUSSIA

Neuropathy target esterase (NTE) is a molecular target for axonopathic OP. This enzyme has proved to be an excellent tool for the assessment of neuropathic potential of OP, in particular, by comparison of an OP inhibitory activity *in vitro* against NTE and AChE. Large-scale OP screening for delayed neurotoxicity was prevented considerably by the lack of an available stable preparation of NTE. To obtain a stable NTE preparation the influence of intensive freezing and subsequent lyophilization of paraoxon-pretreated (P_2+P_3) hen brain membrane fraction on NTE properties has been studied using two neuropathic OP - mipafox and (n-$C_3H_7O)_2P(O)OCH{=}CCl_2$. It was shown, that lyophilization preserved a high NTE specific activity and did not alter the inhibitor characteristics of the enzyme. A long-term storage studies showed that lyophilized NTE preparation retained rather high specific activity during a year and exhibited inhibitory features virtually identical to those of the native enzyme. A comparative studies of an inhibition of the native and lyophilized NTE preparations by a model series of phenyl phosphonates $RO(C_6H_5)P(O)ON{=}CClCH_3$ (R = alkyl) demonstrated a good correlation between the values pI_{50} obtained with both enzyme preparations. The results allow to conclude that the obtained NTE preparation can be used as a standard, stable and a readily available source of NTE for assessment the anti-NTE activity of OP.

Supported by Grants of ISF (NKH000, KH300) and RFBR (96-04-50252)

P 29

ARYL DIARYLPHOSPHINATES AS INHBITORS OF HEN BRAIN NEUROPATHY TARGET ESTERASE

G.Makhaeva, S.Fomicheva, V.Malygin, A.Kharitonov, A.Yarkevich
Institute of Physiologically Active Substances Russian Academy of Science, Chernogolovka,142432 Russia

Phosphinates are one of structural types of acylating inhibitors of Neuropathy Target Esterase (NTE) which can protect against organophosphate induced delayed neurotoxicity (OPIDN) *in vivo*. They produce phosphorylated NTE which is structurally incapable of conversion to aged NTE. The presence of phenyl group in a phosphoryl moiety of OP molecule is known to lead to increasing selectivity for NTE. A series of aryl diarylphosphinates $Ar_2P(O)OC_6H_4$-X (DAP, Ar = Ph, 4-Tol; X = H, 4-CH_3, 4-$N0_2$, 4-Br) was synthesized by interaction of equimolal amounts of diarylphosphinic chlorides and pyridine with phenoles X-C_6H_4OH in benzene. *In vitro* inhibitor potency of DAP to hen brain NTE and acetylcholinesterase (AChE) has been studied. Diphenylphosphinates were shown to be more potent inhibitors of NTE than corresponding di-4-tolyl derivatives. Anti-NTE activity increased with increasing the electronegativity of X: 4-CH_3 < H < 4-Br < 4-NO_2. The studied DAP as a whole were rather weak inhibitors of NTE: k_i = 9.0÷5.3 10^3 $M^{-1}min^{-1}$. Whereas they have negligible anti-AChE activity (k_i < 10 $M^{-1}min^{-1}$). These findings suggest that the most potent NTE inhibitors can be considered as possible protective agents against OPIDN. More detailed studies are required. Supported by RFBR Grant 96-04-50252.

P 30

PROTECTIVE ROLE OF BLOOD AND LIVER ESTERASES IN TOXIC ACTION OF O,O-DIALKYL-S-CARBOETHOXYBROMO-METHYLTHIOL PHOSPHATES

V.Malygin, G.Makhaeva, Institute of Physiologically Active Substances Russian Academy of Science, Chernogolovka,142432 Russia

The interaction of insecticides $(RO)_2P(O)SCHBrCOOC_2H_5$ (DCBP, R = Et, Pr, i-Pr, Bu, Pent, Hex) with mammalian acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and carboxylesterase (CE) was studied. An acute toxicity of compounds to mice was determined. It was found that DCBP were not hydralyzed by CE and inhibited irreversibly all enzymes. A high inhibitor patency of DCBP to CE ($logk_{i\ (CE)} > 8$) may be the reason of their stability to CE-hydrolysis. Anti-AChE activity of DCBP did not change practically with hydrophobicity increasing: $logk_{i(AChE)}$ = 6.00÷6.76. Whereas anti-BChE and anti-CE activity increased with increasing R: $logk_{i(BChE)}$=6.13 (R=Et) and 8.13 (R=Bu). The acute toxicity of DCBP decreased with R increasing and correlated with the efficiency of compounds binding to BChE and CE. To study the role of binding with nonspecific esterases in toxicity lowering the influence of single injections of Et ($logk_{i(AChE)}$= 6.24, LD_{50} 25 mg/kg) and Bu-DCBP ($logk_{i(AChE)}$= 6.60, LD_{50} 162 mg/kg) to blood and liver esterase activity has been studied. The i/p administration of Et and Bu-DCBP in doses 0.15 LD_{50} was shown to lead to fast and substantial CE and BuChE inhibition in blood and liver, the Bu-DCBP inhibited both enzymes to a greater degree. The results indicate to buffer, protective role of nonspecific esterases in toxic action of DCBP and explain largely the lower toxicity of Bu in comparison to Et derivative.

Supported by RFBR Grant 96-04-50252

P 31

GLUCOCORTICOIDS AND ELECTROMECHANICAL ACTIVITY INDEPENDENTLY REGULATE ACETYLCHOLINESTERASE IN THE MAMMALIAN SKELETAL MUSCLE.

J. Vodiškar, V. Zgonc, S. Ribarič, and Z. Grubič
Institute of Pathophysiology, School of Medicine, University of Ljubljana, 1000 Ljubljana, Zaloška 4, Slovenia

Glucocorticoid (GC) and electromechanical control of acetylcholinesterase (AChE) expression in mammalian skeletal muscle are well documented. Both, low-frequency stimulation (slow muscle pattern) and GC treatment downregulate AChE activity in the adult fast rat skeletal muscle. However, the mechanisms underlying the effects of these treatments seem to be different. In the GC treated muscle, we observed decreased AChE activity at the unchanged AChE mRNA level (Brank et al., *Eur.J.Biochem,* 1997, in print). On the other hand, electromechanical activity seems to exert its effect primarily at the mRNA level (Jasmin et al. *Neuron* 11, 467-477, 1993; Grubič et al., *Neuron* 14, 317-327, 1995). If GCs and electromechanical activity indeed act at different levels of AChE biosynthetic pathway, one could hypothesize that their effects are independent on each other and that no mutual influences could be expected among both treatments. In order to test this hypothesis, we studied the effects of combined treatment (GCs and electrical stimulation - tonic pattern) on the AChE activity levels in the fast rat EDL muscle. AChE activities were determined and compared among the following experimental groups of muscles: 1) control, nontreated 2) dexamethasone-treated-only, 3) electrically stimulated-only (tonic pattern), and 4) dexamethasone-treated and simultaneously electrically stimulated, muscles.

Decrease in AChE activity due to GC treatment was practically the same in GC-treated-only muscles and muscles that were GC-treated and simultaneously electrically stimulated. Additive effects of both treatments support the hypothesis that GCs and electromechanical activity regulate AChE metabolism in the fast rat skeletal muscle at different levels.

P 32

GLUCOCORTICOIDS DIFFERENTIALLY CONTROL SYNTHESIS OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE IN RAT LIVER AND BRAIN.

U. Weber, M. Brank, and Z. Grubič
Institute of Pathophysiology, School of Medicine, University of Ljubljana, 1000 Ljubljana, Zaloška 4, Slovenia

Mammalian organisms posses two cholinesterases: acetyl- (AChE) and butyryl- (BuChE) cholinesterase. In spite of numerous theories and proposals, we still have no conclusive explanation for this dual representation of acetylcholine-hydrolysing enzymes. One approach to investigate the function of the two enzymes is to study their responses to specific and well defined physiological or pharmacological factors. In the present study we aimed to elucidate the glucocorticoid (GC) influence on the metabolism of AChE and BuChE in rat brain and liver. Female Wistar rats were daily injected with dexamethasone (4,5 mg/kg b.w.) until their body weight dropped for >15%, signaling fully expressed GC effects. At this stage, we determined AChE and BuChE activities in the homogenates of isolated liver and brain. A new technique, based on the precise radiometric measurements of AChE and BuChE activities in the polysomic fractions, prepared from liver and brain under the nondenatured conditions, was used to study GC effects on the early stages of synthesis of both enzymes.

We found significantly reduced BuChE activity in both, brain (- 30,3 %) and liver (- 60,7 %) after dexamethasone treatment. AChE activity in brain remained practically unchanged ($p > 0,05$), while liver AChE activity decreased significantly (- 18,8 %) These results, together with the GC-mediated elimination of the correlation between BuChE and AChE activities in brain and liver, support the theory, proposed by Edwards and Brimijoin (*J Neurochem,* 38: 1393-1403, 1982, and *Biochem Pharmacol,* 32: 1183-1189, 1983) that BuChE activity is regulated by systemically acting factors, while the regulation of AChE activity is primarily tissue-specific.

P 33

DEGRADATION RATES OF AChE mRNA IN THE NORMAL AND DENERVATED RAT FAST SKELETAL MUSCLE, STUDIED *IN VITRO.*

K. Zajc-Kreft, S. Kreft, and Z. Grubič
Institute of Pathophysiology, School of Medicine, University of Ljubljana, 1000 Ljubljana, Zaloška 4, Slovenia

Well documented downregulation of acetylcholinesterase (AChE) mRNA in the skeletal muscle by electromechanical activity is in seeming contradiction with the decreased transcript level observed after denervation of rat muscle. One could hypothesize that electromechanical activity downregulates AChE in the extrajunctional region - predominating in every muscle - in a way, similar for all synaptic proteins, while denervation decreases AChE message through some other events, efficient enough to override the expected upregulating effects of electromechanical inactivity. One explanation for the decreased level of AChE mRNA in the denervated muscle would be increased mRNA degradation capacity under such conditions. In order to test this possibility, we exposed total deproteinated RNA, isolated from rat m. sternomastoideus (m.SM), to subcellular muscular fractions prepared from either normal or denervated contralateral m.SM. After selected time intervals, we determined remaining AChE mRNA by Northern blotting.

AChE mRNA remained at the same level during the first 5h after denervation and fell dramatically after subsequent 13 h. Increased degradation rate of AChE mRNA was observed in subcellular fractions isolated from the denervated muscle. Postmitochondrial and postpolysomal fractions exhibited higher degradation rate than polysomal fraction. α-actin mRNA behaved similarly, suggesting nonspecific AChE mRNA degradation Longer transcript (3.5 kb) was more affected than the shorter (2.3 kb). The above results suggest, that in the denervated rat fast muscle, degradation rate is increased for a certain group of mRNAs, including AChE mRNA. This finding could at least partly explain decreased level of AChE mRNA under such conditions.

P 34

MYONUCLEAR DENSITY AND DISTRIBUTION IN THE NORMAL AND DENERVATED RAT FAST SKELETAL MUSCLE.

T. Milanič, A. Kunstelj, T. Marš, and Z. Grubič
Institute of Pathophysiology, School of Medicine, University of Ljubljana, 1000 Ljubljana, Zaloška 4, Slovenia

Unlike majority of mammalian tissues, having mononuclear cells as basic units of their tissue composition, skeletal muscle fiber is a polynuclear syncytium. Such organization offers additional possibility for the regulation of expression of various proteins including cholinesterases. Besides various stages of protein synthesis, expression can also be controlled at the level of myonuclear, i.e. gene distribution. Higher or lower concentrations of genes in the region might have important impact on the expression of proteins in this region, since it is known that expression can also depend on the local concentrations of certain gene products. The aim of our study was to test, if morphometric parameters of the myonuclear distribution in the adult rat muscle change after denervation. Such changes would allow the explanation that some effects of denervation originate from the events taking place at the level of myonuclear redistribution. Myonuclei were fuorescently stained and their distribution was morphometrically analyzed in the single muscle fibers isolated from the normal and denervated rat fast m. sternomastoideus.

Synaptic concentration of myonuclei was found to exceed extrasynaptic concentration by the factor of 17. Extrasynaptic nuclei were significantly ($p<0.05$) longer than the synaptic ones. No significant changes in the number of nuclei accumulated at the endplates or in their morphometric criteria were observed one week after denervation. However, extrasynaptic nuclei became more concentrated and smaller ($p<0.05$). Insignificant ($p>0.05$) rise in total RNA per muscle and significantly increased total RNA per weight muscle (+24%) were observed under such conditions. Total RNA per myonucleus was estimated to be decreased. Causal relationship between the observed events and other postdenervational muscle alterations remains unknown.

P 35

LOCALIZATION OF CELLS EXPRESSING AChE mRNA IN RAT BRAIN USING NONRADIOACTIVE *IN SITU* HYBRIDIZATION COMBINED WITH FLUORESCENT NUCLEAR LABELING

K. Miš, K. Zajc-Kreft, and Z. Grubič
Institute of Pathophysiology, School of Medicine, University of Ljubljana, I000 Ljubljana, Zaloška 4, Slovenia

Better understanding of the role of AChE in mammalian brain necessitates the knowledge on the distribution of AChE synthesizing cells in this tissue. The aim of the present study was to test a novel, nonradioactive approach for the localization of AChE mRNA positive cells in striatum, cortex and hippocampus of the rat brain. AChE mRNA was localized by nonradioactive *in situ* hybridization, which has not been used previously for the localization of this message in mammalian brain. In order to find optimal conditions for localization, we tested both, oligonucleotide and RNA probe. We also tested various prehybridization protocols and approaches. Total number of cells in brain regions was determined by subsequent fluorescent staining of nuclei in the same brain section.

Best AChE mRNA localization was obtained by digoxigenine-labelled RNA probe. Acetylation step prior to hybridization was found essential for the optimal signal/background ratio. High nonspecific staining was observed if this step was omitted. In accord with other authors (Landwehrmeyer et al., *Neuroscience,* 57: 615-634, 1993; Hammond et al., *PNAS,* 91: 10933-10937, 1994; Bernard et al., *Neuroscience,* 64: 995-1005, 1995), who used radioactive in situ hybridization, we found the lowest portion of AChE mRNA - positive cells in striatum. Hippocampus (CA region), and cortex had higher portions of AChE mRNA - positive cells, which, however, exhibited lower intensity of transcript staining. In comparison to radioactive techniques, nonradioactive approach proved much faster (the whole procedure, from fixation to staining, is completed in two days) and easier to work with, while the results are practically the same.

P 36

Chemical and physical studies of acetylcholinesterase inhibition by its naturally occurring inhibitor, poly-APS

Kristina Sepčić[1], Nataša Poklar[2], Gorazd Vesnaver[2], Tom Turk[1] and Peter Maček[1]

[1]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1111 Ljubljana, Slovenia
[2]Department of Chemistry, University of Ljubljana, Aškerčeva 5, P.O. Box 537, 1000 Ljubljana, Slovenia

Poly-APS, polyalkylpiridinium salts isolated from the marine sponge *Reniera sarai,* act as potent cholinesterase inhibitors. They show an unprecedented inhibition kinetics that is composed of several successive phases. Previous kinetics studies have shown that the initial binding of poly-APS molecule to the surface of the enzyme occurs at the peripheral anionic site. This reversible binding is followed by a slow binding and/or irreversible inhibition, resulting in an irreversible block of AChE activity and denaturation of the enzyme. To provide more information about inhibitor-enzyme interactions, we used different fluorimetric techniques and electrophoresis. All experiments were performed using wild type recombinant insect acetylcholinesterase to avoid contamination with other proteins. CD-measurements in far- and near-UV range showed significant changes in AChE secondary structure when enzyme was titered with poly-APS. We found a 40% increase of unordered structure, after which the enzyme precipitated. The enzyme precipitation was proved by SDS-PAGE. We also observed a decrease of intrinsic Trp fluorescence when AChE was titered with poly-APS. A k_{bind} derived from these measurements was about 40 nM and thus in accordance with our previous kinetics results, in which we calculated the value of 11 nM for the initial, reversible binding of poly-APS to the peripheral anionic site. The enzyme-inhibitor interaction could be monitored spectrofluorimetrically as an increase of light scattering due to aggregation of protein. Finally, there is evidence that poly-APS can bind non-specifically to several other proteins.

P 37

PARAOXONASE POLYMORPHISM IN RABBITS

Ronald Zech, Ragna M. Severin, Jörg M. Chemnitius[a], Klaus Nebendahl[b]
Department of Biochemistry, Department of Cardiology[a] and Department of Experimental Animal Research[b], Georg-August-University, D-37075 Göttingen, Gerrnany

Paraoxonase in serum and liver of rabbits and cattle was investigated. In serum the two substrates paraoxon and phenylacetate are exclusively hydrolyzed by α-lipoprotein-bound paraoxanse. In rabbit liver paraoxon is hydrolyzed only by paraoxonase, while phenyl acetate is hydrolyzed by paraoxonase (20%) and additionally by an organophosphate sensitive carboxylesterase (B-Esterase), which is responsible for 80% of total liver phenyl acetate hydrolysis. Phenylacetate hydrolysis by B-Esterase of rabbit liver was shown to be inhibited by paraoxon and by mipafox covalently in a time and concentration dependent manner. Rabbit serum exhibits by far the highest serum paraoxonase activity (2.6 ± 0.6 U/ml) of all vertebrate species tested up to now, while rabbit liver contains only 0.5 ± 0.2 U per grams fresh weight. In cattle extremely high paraoxonase activity is found in liver (2.8 U/g), while bovine serum contains only 0.2 U/g,

The paraoxanase activity ratio (hydrolysis rate paraoxon : phenytacetate x 1000) in cattle does not show interindividual variation (activity ration 4.0 ± 0.4, correlation coefficient 0.996, $p < 0.001$). In contrast, the paraoxon/phenylacetate hydrolysis ratio of rabbit paraoxonase in serum as well as in liver does vary considerably between individuals. In crossbred rabbits paraoxonase activity ratios from 3 to 10 are found. In a strain of pure bred New-Zealand-White rabbits three polymorphic serum paraoxonase phenotypes could be clearly differentiated by the activity ratio. In analogy to human paraoxonase polymorphism the rabbit paraoxonase isotypes where classified as paraoxonase A (activity ratio 3.8 - 4.3), AB (ratio 5.5 - 6.0) and B (ratio 7.3 - 8.6). The corresponding frequencies of' the three isotypes were 40, 35 and 25%.

P 38

MOLECULAR POLYMORPHISMS OF HUMAN SERUM PARAOXONASE (PON) AFFECT ACTIVITY

B. Mackness, M.I. Mackness, S. Arrol, W. Turkie, P.N. Durrington
University Department of Medicine, Manchester Royal Infirmary, Manchester, U.K.

Molecular polymorphisms of the PON1 gene give rise to amino acid substitution at position 55 (Met (M)→Leu (L)) and 192 (Gln(A)→Arg(B)). The 192-polymorphism affects the hydrolysis of organophosphate substrates such as paraoxon (hydrolysed more rapidly by B-alloenzyme) and diazoxon (hydrolysed more rapidly by A-alloenzyme). The PON-55 polymorphism also influenced PON1 activity. The MM genotype was associated with significantly lower activity towards paraoxon than the LL and LM genotvpe ($P<0.001$) independent of the PON-192 polymorphism.

The 192 polymorphism may also affect the anti-atherogenic properties of PON. The AA alloenzyme is more effective at preventing lipid-peroxide generation on LDL (40% protection at 6 hrs), than either of the AB alloenzymes (23% protection, $P<0.025$) or the BB alloenzyme (1% protection, $P<0.005$) which may explain recent associations between the PON genetic polymorphism and CAD in some epidemiological studies. The effect of the PON polymorphism on the anti-atherogenic properties of PON is currently under investigation.

P 39

IMMUNOLOCALISATION OF PARAOXONASE WITH CLUSTERIN AND APOLIPOPROTEIN A1 IN THE HUMAN ARTERY WALL

B. Mackness, R. Hunt, P.N. Durrington and M.I. Mackness
Depts of Medicine and Pathology, University of Manchester, The Royal Infirmary, Manchester M13 9WL, U.K.

HDL associated paraoxonase (Pon) can prevent the accumulation of lipid-peroxides on LDL incubated under oxidising conditions *in vitro.* Pon is associated with a specific HDL particle also containing clusterin and apolipoprotein A1 (apo A1) and it has been suggested that this HDL particle may have a specific role as a membrane and also lipoprotein protective agent by preventing lipid-peroxidation. We investigated the co-immunolocalisation of Pon, clusterin and apo A1 in the wall of normal and atherosclerotic human aortas. In normal aorta's (n=6) there were low levels of extracellular Pon, apo A1 and clusterin. The cytoplasm of smooth muscle cells in the media showed granular positivity for both Pon and apo A1 indicating that these proteins were undergoing lysosomal degradation. This was also indicated by the presence of both intact and degradation products of Pon in smooth muscle cells as shown by Western blotting. With progression of disease from fatty streaks (n=3) to advanced atherosclerosis (n=8) there was an increase in extracellular Pon, apo A1 and clusterin indicating accumulation of these proteins in atherosclerotic tissue.

HDL components are therefore found to increase in the artery wall with the progression of atherosclerosis and may be participating in the clearance of damaged and necrotic tissue.

P 40

PARAOXONASE AND ARYLESTERASE ACTIVITIES IN THE SERUM OF TWO HYPERLIPOPROTEINAEMIC PATIENTS AFTER REPEATED EXTRACORPORAL LIPID PRECIPITATION

E. Reiner, D. Svedružić, V. Simeon-Rudolf *(Institute for Medical Research and Occupational Health)* **and V. Lipovac, M. Gavella, V.Mrzljak** (V. *Vrhovac Institute, University Clinic, Medical Faculty) Zagreb, Croatia*

The effect of heparin-induced extracorporal lipid precipitation (HELP) on the activities of total paraoxonase (EC 3.1.8.1) and of EDTA-sensitive *(v-sen)* and EDTA-insensitive (*v-ins*) arylesterase (EC 3.1.1.2) was studied in serum of a patient with hyperlipoproteinaemia (A) and in a patient with non-insulin dependent diabetes mellitus and hyperlipoproteinaemia (B). The enzyme activities were measured spectrophotometrically (Tris/HCI buffer, pH=7.4, 37°C) with paraoxon and phenylacetate as substrates of paraoxonase and arylesterase resp. Both patients underwent HELP applications once a week over a period of 7 weeks. Over that period no overall change was observed either in enzyme activities or in the lipid or protein content of the serum samples. However, each HELP session caused an immediate decrease of *v-ins* (on average 56% in A and 42% in B), while *v-sen* arylesterase remained almost unaltered. Paraoxonase remained unchanged in A, but decrease in B (about 60%). Of the atherogenic parameters, the most pronounced decrease was found in VLDL-cholesterol and in triglycerides (on average 45% in A and 32% in B), while the anti-atherogenic HDL-cholesterol decreased less than 10%. Possible implications of the effect of HELP on the studied enzyme activities remain to be explained.

P 41

PARAOXON (POX) INHIBITS PHOSPHOLIPASE A_2 (PLA_2)

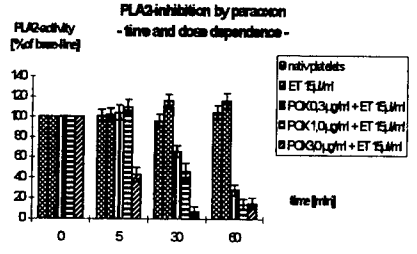
Helfrich U, Schmitt A, Toomes M, Petroianu G, Ruefer R;
University of Heidelberg at Mannheim; Dept. of Pharmacology & Toxicology; 14-16 Maybach Street; 68169 Mannheim - Germany.

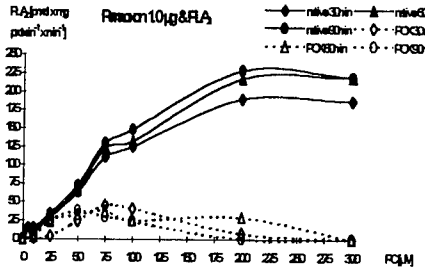
INTRODUCTION: POX, an esterase inhibitor, also inhibits the PLA_2. [*J Appl. Toxicol. 1997*].

PURPOSE: To establish the dose/time-dependence of the POX induced inhibition of the activated PLA_2-molecule.

MATERIAL & METHODS: PLA_2 is incubated for 5, 30 and 60 min with TRIS-buffer (native), ethanol (ET) 15 µl and POX 0.3, 1.0 and 3.0 µg/ml dissolved 15 µl ET respectively. PLA_2-activity is measured in a radioactive assay. Base-line values are considered to be 100%; all other values are expressed as percentage thereof. Data is analysed with the Mann Whitney test. Significance is assumed for $p \leq 0.05$ (Fig A). Figure B shows PLA_2 activity at different substrate concentrations in the presence or absence of POX (1.0 µg/ml) after different incubation times.

RESULTS: **A** **B**





DISCUSSION: POX inhibited in a dose & time dependent manner the activated PLA_2-molecule. Lineweaver-Burk representation of the data suggests an uncompetitive inhibition.

P 42

ACETYLCHOLINE INHIBITS PLA_2 ACTIVITY.

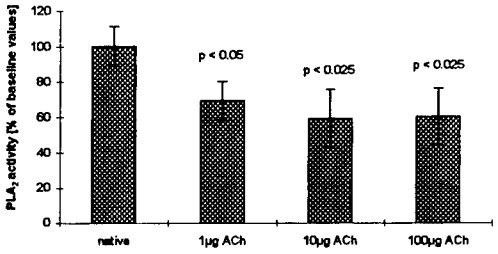
Helfrich U, Petroianu G, Bergler W, Fisher J, Ruefer R;
University of Heidelberg at Mannheim; Department. of Pharmacology & Toxicology; Maybach Street 14-16; 68169 Mannheim-Germany.

Paraoxon (POX) causes an inhibition of acetylcholine (ACh)-esterase with subsequent increase in ACh concentrations. POX also inhibits phospholipase A_2 (PLA_2) activity.

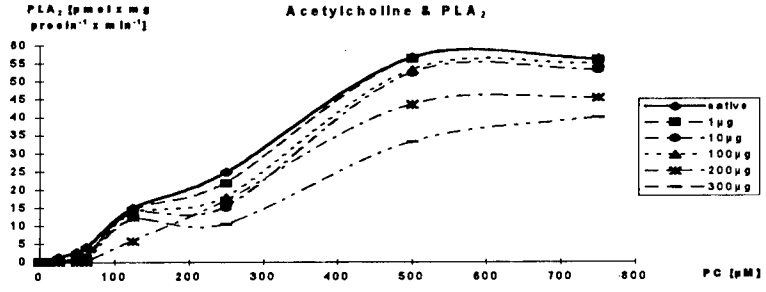
PURPOSE OF THE STUDY: To establish the influence of ACh on the PLA_2 activity in an in vitro model.

MATERIALS & METHODS: PLA_2 is incubated with TRIS-buffer or 1, 10 and 100µg ACh respectively. Activity is measured by radioactive assay. All values are expressed in % of baseline values (100%). Data are analysed with the rank order test. Statistical significance is assumed for $p \leq 0.05$.

RESULTS:



DISCUSSION: The PLA_2 activity reduction after POX exposure is due not only to POX but also to endogenous Ach. Lineweaver-Burk representation of the data suggests a non-competitive inhibition.



P 43

PHOSPHOLIPASE A_2 (PLA_2) ACTIVITY IN MINI PIGS AFTER ACUTE PARAOXON (POX) INTOXICATION.

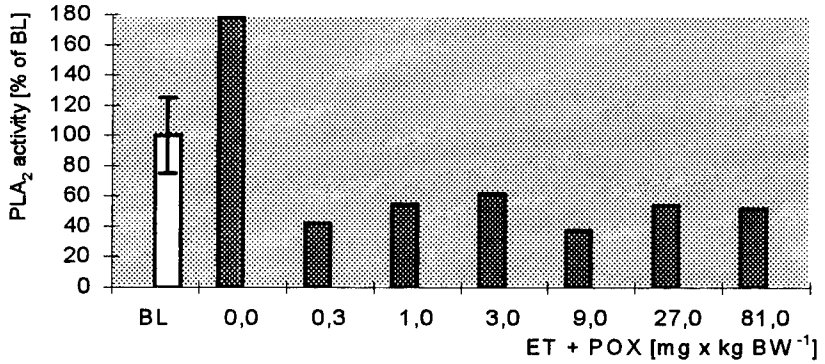
Petroianu G, Helfrich U, Fisher J*, Bergler W, Ruefer R;
University of Heidelberg at Mannheim, Department of Pharmacology & Toxicology; Maybach Street 14-16; 68169 Mannheim-Germany.
*Toronto Hospital, Ontario, Canada

INTRODUCTION: POX, a serine enzyme inhibitor, also inhibits PLA_2 activity in vitro in a dose dependend manner (J Appl Toxicol 1997).

PURPOSE:: To establish the dose dependency of PLA_2 inhibition by POX in acutely poisoned minipigs.

MATERIALS & METHODS: Seven anaesthezised minipigs are infused POX in ethanol over 50 min. Measurments are carried out before (baseline) and after POX-infusion. PLA_2 activity is measured in a radioactive assay. All values are expressed in % of baseline values (100%).

RESULTS: POX infusion causes a significant reduction in PLA_2 activity. No dose dependency can be observed.



DISCUSSION: Many drugs used during anaesthesia affect PLA_2 activity [Arch Pharmacol 356 (Suppl.1) R28]. They might mask the dose dependency of inhibition due to POX.

P 44

PARAOXON DOES NOT INHIBIT ß-LACTAMASE.

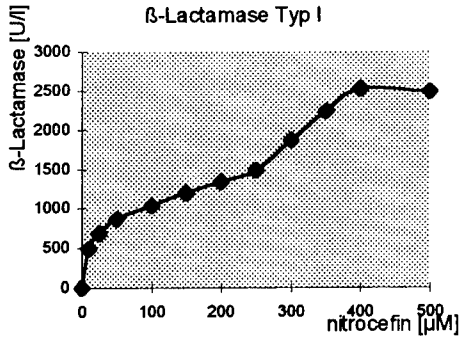
G Petroianu, U Helfrich, M Toomes, R Ruefer; Dept. of Pharmacology & Toxicology; Maybach Street 14-16; 68169 Mannheim-Germany.

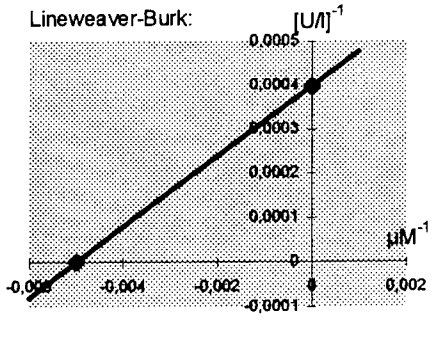
INTRODUCTION: Paraoxon (POX), a serine hydrolase inhibitor, might affect enzymes (ßLAC) hydrolyzing ß-lactam antibiotics.

PURPOSE OF THE STUDY: To establish the influence of POX on ßLAC.

MATERIALS & METHODS: ßLAC typ I & III (Bacillus cereus), typ II & IV (Enterobacter cloacae) and RTEM (E. coli) were incubated with POX in ethanol (ET) or ET alone. ßLAC activity was measured using the chromogenic substrate nitrocefin. Native activities = 100%; all other values % thereof.

RESULTS	ET 15µl	POX 0.3µg	POX 1.0µg	POX 3.0µg
Typ I	102 ± 7	120 ± 8	110 ± 25	105 ± 2
Typ II	100 ± 14	100 ± 15	99 ± 15	98 ± 15
Typ III	107 ± 17	97 ± 14	99 ± 15	91 ± 13
Typ IV	119 ± 20	121 ± 22	122 ± 21	106 ± 22
RTEM	103 ± 12	103 ± 12	126 ± 19	112 ± 13





CONCLUSION: No inhibitory effect of POX on ßLAC activity was noticed. The addition of serine hydrolase inhibitors to antibiotic therapy is not usefull.

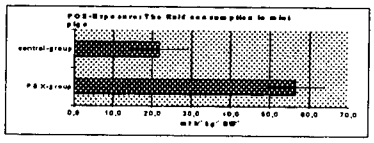
P 45

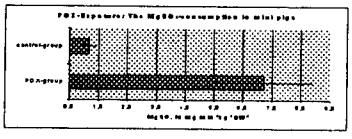
CONTROL OF BLOOD PRESSURE (BP), HEART RATE (HR) AND HEMATOCRIT (Hct) DURING HIGH DOSE IV-PARAOXON (POX) EXPOSURE IN MINI PIGS. M. Toomes, G. Petroianu, W. Bergler, A. Petroianu, R. Rüfer; University of Heidelberg, at Mannheim; Maybach St. 14 - 16; 68169 Mannheim - Germany

Introduction: Without agressive therapy high dose POX is lethal. The animals die shortly after POX-infusion due i.a. to hypertonus, tachycardia and an increase in hematocrit.

Purpose of study: To asses the acute hemodynamic effects of high dose POX exposure in mini pigs indirectly by the requirements of antihypertensive- and fluid-therapy.

Material and methods: Anesthesized mini pigs received iv-POX (54 mg kg^{-1} BW^{-1}) in alcohol. The control group received alcohol in corresponding amounts. When the BP and HR increased, $MgSO_4$ titrated to effect was given iv until BP/HR reached normal range. When the Hct increased, fluids were given iv until Hct reached normal range. The measured values in the two groups were compared by the rank order test (Mann & Whitney, 1947).





Results: As planned, no statistically significant differences concerning BP, HR or Hct were found. The POX-group however required significantlly more $MgSO_4$ and fluids than the control group in order to keep BP, HR, and Hct within normal range.

Discussion: We assume that the increase of BP and HR is due to a pheochromocytoma-like pattern caused by an excessive release of catecholamines from the adrenal medulla. The fluid consumption may be due to a direct toxic effect of POX on biological membranes (Antunes-Madeira, 1994). An extravasation due to damaged endothelial cells seems probable. An activation of secretory glands contributes to an increase in Hct due to consumption of fluids.

P 46

HIGH DOSE INTRAVENOUS PARAOXON (POX) EXPOSURE: COAGULATION STUDIES IN MINI PIGS

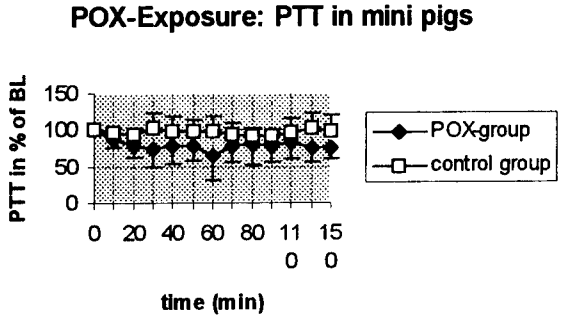
M. Toomes, G. Petroianu, W. Maleck, W. Bergler, R. Rüfer; University of Heidelberg, at Mannheim; Maybach St. 14 - 16; 68169 Mannheim -Germany

Introduction: Previous in vitro studies showed no coagulation activating effect of POX to human blood (Petroianu, 1996). No satisfactory explanation has been provided to date for the alterations of blood coagulation seen in vivo due to organophosphourus compounds.

Purpose of the study: Establish the in vivo effects of POX on the coagulation of mini pigs as assessed by measurements of the partial thromboplastin time (PTT), prothrombin time (PT), fibrinogen, factor V, factor VII, factor VIII, antithrombin III, protein C, and platelet count.

Methods and materials: Anesthsized mini pigs were randomly assigned to the POX-group (n=9) receiving 54 mg POX $kg^{-1}BW^{-1}$ and a control group (n=9). Measurements were carried out over a period of 480 minutes after poisoning. Statistical analysis was performed using the Whitney-Mann test.

Results: No changes were seen during the 480 min of observation in PT, factor V, VII, VIII, antithrombin III, protein C or fibrinogen. PTT was shortened early in the observation period (t = 10 -60 min).



Discussion: The hypercoagulability seen in the early „sympathotone" phase (Roth, 1993) is probably due to a massive release of catecholamines from the adrenals and not a direct effect of POX on the coagulation system.

P 47

DIFFERENTIAL SEQUESTRATION OF TWO ORGANOPHOSPHORUS HOMOLOGS BY THE RAT LIVER HOMOGENATE

P. Santhoshkumar and T. Shivanandappa
FP&IC Department, Central Food Technological Research Institute Mysore - 570 013. India.

Bromophos and ethylbromophos are two structurally homologous organophosphorus insecticides (OP) which show 24-fold difference in their toxicity to laboratory rat (LD_{50} - 91 and 2215 mg/kg b.w. respectively). The ability of the rat liver carboxylesterases (CaE) to sequester the oxons of these OP was studied *in vitro*. Both bromoxon (Bo) and ethylbromoxon (EBo) are greater inhibitors of rat hepatic CaE than brain acetylcholinesterase (AChE) with IC50 values at nanomolar and picomolar levels, respectively. The capacity of the liver CaE to sequester OPs was determined by measuring the brain AChE inhibition pre-incubated with or without liver homogenate. AChE inhibition by Bo decreased with increasing concentration of liver tissue. Preincubation of the oxons with the liver extract (CaE) decreased the degree of AChE inhibition in the case of Bo whereas it was unaffected in the case of EBo. The results imply that liver tissue contains binding sites which sequester Bo significantly thereby reducing the number of OP molecules available to inhibit AChE. As the IC5O value of EBo to hepatic CaEs is 50-100 times lower than that of Bo, the concentration of former oxon used in the assay could be sufficient to inhibit brain AChE after inhibiting CaE activity. Thus differential sequestration of the OPs by hepatic CaE could be important in understanding the role of different saturation of the target molecules and their consequence to the expression of neurotoxicity.

P 48

CHARACTERIZATION OF A SOLUBLE MOUSE LIVER ENZYME CAPABLE OF HYDROLYZING DFP (DIISOPROPYL PHOSPHOROFLUORIDATE)

S.S. Billecke, S.L. Primo-Parmo, C.S. Dunlop, J.A. Doorn, and B.N. La Du, Depts. of Pharmacology and Anesthesiology, University of Michigan Medical School, Ann Arbor, MI 48109-0615, and C.A. Broomfield, Biochemical Pharmacology Branch, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425

In our search for PON1-like enzymes in mammalian tissues, we sought candidates with DFPase activity in the soluble fractions of mammalian livers. A novel mouse liver DFPase was purified and characterized which also has organophosphatase activities with sarin, soman and tabun, but lacks paraoxonase and arylesterase activities with paraoxon and phenyl acetate, respectively. This soluble mouse DFPase resembled that purified by Little *et al.* (*Biochem. Pharm.* **38**, 23-29, 1989) from the soluble fraction of rat liver in regard to its substrate specificity, size (about 39 kDa) and its stimulation by several metal ions, namely magnesium, manganese and cobalt.

We purified the mouse liver enzyme using an improved DFPase method based on the rate of acid production under standardized conditions with Phenol Red as an indicator. After ion exchange chromatography, adsorption and elution from a hydrophobic gel (phenylsepharose) and a Sephacryl column, one major protein band was observed in SDS PAGE gels. This band was sequenced after cyanogen bromide cleavage and found to be identical in its amino acid sequence with the recently identified senescence marker protein-30 (SMP-30) of Fujita *et al.* (*Biochim. Biophys. Acta* **1308**, 49-57, 1996). Senescence marker proteins possessing high structural homology with the mouse SMP-30 have also been found in human and rat livers. Of the mouse SMP-30's 299 amino acids, 10 are cysteine residues. The DFPase activity is inhibited by p-hydroxy-mercuribenzoate but not by iodoacetate. To date, the only promising substrate other than DFP is sodium 4-[(5-methyl-2-oxo-1,3-dioxol-4-yl)methylthio]-benzene-sulfonate 1/2 hydrate, which is also a substrate for purified human serum PON1. No activity was found with α and β naphthylacetates, 7-acetoxy-N-methylquinolinium iodide, 7-acetoxy-3-cyanocoumarin 4-methylumbelliferyl acetate and 4-methylumbelliferyl butyrate, all substrates for human PON1. A zymogram has shown that DFPase activity is located in the same region as the sequenced protein band but to be sure that the SMP-30 and this DFPase are identical the recombinant enzyme will be expressed and tested. The expressed enzyme will also facilitate our substrate specificity studies.

The sequences of mouse SMP-30 and the three PON family members show no significant structural homology. Thus, if the recombinant SMP-30 has DFPase activity, it will be clear that at least two distinct, non-homologous families of mammalian liver enzymes have DFPase activity.

P 49

DISTRIBUTION PROFILES OF PARAOXONASE AND CHOLINESTERASE PHENOTYPES IN A SPANISH POPULATION

A.F. Hernández, M.C. Gonzalvo, F. Gil, L. Rodrigo, E. Villanueva and A. Pla.
Department of Legal Medicine Legal and Toxicology Service. Faculty of Medicine. University of Granada. Spain.

It has been reported that interindividual differences in the activity of paraoxonase and cholinesterase may contribute to variations in susceptibility of man to the toxic effect of pesticides, especially organophosphates (OP). Both enzyme activities have been shown to vary within the healthy population and to be under genetic control. The paraoxonase and cholinesterase were analysed in the serum of 184 blood donors from Granada (Southeastern Spain). Ratios of paraoxonase activity in the presence of 1M NaCl divided by the arylesterase activity were used to identify individual phenotypes. The gene frequencies for the low activity allele (A) was 0.63 and for the high activity allele 0.37. Thus, 47.8% of this population was homozygous for the low activity allele (AA), 21.7% were homozygous for the high activity allele (BB), and 30.4% were heterozygous (AB). These frequencies are close to those observed in Caucasian samples from North America and Europe. Phenotypic frequencies in the sample fit the Hardy-Weinberg equilibrium. Cholinesterase was phenotyped by measuring the inhibition of benzoylcholine hydrolysis with dibucaine and fluoride. 26 individuals (13.4%) had baseline measurements below the normal reference interval for the usual (UU) phenotype and only 2 were below de lower limit for the US (usual/silent) phenotype. From the total sample, 89.7% had the usual (UU or US) phenotype while the other 10.3% were heterozygous for the atypical deficiency (UA); and 4 (2.1%) were heterozygous for the UF (usual/fluoride-resistant) phenotype. Nevertheless, no homozygous for the atypical or silent phenotypes (the most susceptible when exposed to organophosporus pesticides) were observed in the above group. 42 individuals living in an intensive agricultural area at the seaside of Granada, but not exposed to pesticides were also phenotyped for the same parameters, showing similar results. We consider it useful to incorporate these two biomarkers into the health programme of greenhouse workers with the purpose of monitoring workers who spray OP pesticides as they provide reliable indications of early-stage effects related to biochemical alterations that might precede overt clinical pictures.

P 50

BIOLOGICAL VARIATIONS OF SERUM CHOLINESTERASE ACTIVITY IN POPULATION OF ZAGREB, CROATIA

ŠIFTAR Z., FLEGAR-MEŠTRIĆ Z., ŠURINA B.

INSTITUTE OF CLINICAL CHEMISTRY, CLINICAL HOSPITAL "MERKUR", ZAJČEVA 19, ZAGREB, CROATIA

Biological variations in total cholinesterase activity (EC 3.1.1.8) in sera were studied in a population of 600 healthy school children (age 8-19 years) and 120 healthy adult person from population of Zagreb, Croatia.

Variations according to age and sex, morphometric parameters and genetically determined changes were studied as the most important factors affecting biological variation in total cholinesterase activity.

In order to specify the contributions of genetic and physiological characteristics to biological variations in the total cholinesterase activity phenotyping was done using butyrylcholine as substrate (1) and dibucaine, fluoride, urea and Ro 02-0683 as inhibitors. The following phenotypes were obtained: UU,UA,UF, AK and AA with associated frequency of 94.4, 4.7, 0.3, 0.3 and 0.5 respectively.

Based on biological variability, using non-parametric statistical method (2), reference intervals for total cholinesterase activity for the population of Zagreb, Croatia, aged 8-70 years, were produced in order to provide medically reliable transversal evaluation of laboratory results.

1. Ann. Clin. Biochem.1985; 22:176-178.
2. Arch Pathol Lab Med 1992; 116:710-713.

P 51

ORGANOPHOSPHATE TOXICITY TO MAN - INTER INDIVIDUAL DIFFERENCES IN SUSCEPTIBILITY

Faith M Williams and Elaine Mutch. Toxicology Unit, Department of Occupational and Environmental Medicine, The Medical School, Newcastle University, NE2 4HH, UK. *F.M. Williams@ncl.ac.uk*

Organophosphorous compounds are acutely toxic to man by inhibition of acetylcholinesterase in the nervous system. Repeat exposure to low levels of organophosphates may also lead to a delayed toxicity. Differences in expression of target enzyme and of activation and detoxification enzymes may contribute to interindividual differences in susceptibility to organophosphate exposure in man. Many pesticides are phosphorothioates which require activation by cytochromes P-450 to the toxic oxon before binding to acetylcholinesterase. Organophosphates are rapidly removed from the circulation and inactivated by hydrolysis by paraoxonase or binding to non-specific esterases. Our studies have shown that parathion (a phosphorothioate) is activated to the toxic oxon (paraoxon) and detoxified to *p*-nitrophenol in parallel by cytochrome P-450, mainly CYP3A, in human liver microsomes in an *in vitro* system(sixteen fold range). CYP3A exhibited considerable inter individual differences in expression due to environmental influences on CYP3A4 and the presence or absence of CYP3A5 which may be under genetic control in man.

Plasma paxaoxonase activity ranged eight fold in a UK Caucasian population. The relative roles of paraoxonase hydrolysis, carboxylesterase binding and CYP mediated detoxification pathways in the liver have been defined by comparing the metabolic profile with and without EDTA (a paraoxonase inhibitor). Substrate concentrations have been selected to define metabolic profiles at parathion levels encountered *in vivo* compared to saturation substrate conditions. At low parathion concentrations p-nitro phenol formation by liver microsomes does not involve paraoxonase.

Factors specific to an individual, such as genetic makeup as well as the type of exposure, may influence the toxicokinetics and hence toxicity of phosphorothioate pesticides.

P 52

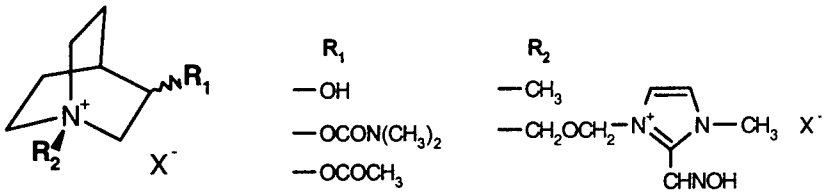
3-HYDROXYQUINUCLIDINIUM DERIVATIVES: SYNTHESIS AND STRUCTURES

Ines Primožič, Renata Odžak and Srđanka Tomić

Department of Chemistry, Faculty of Science and Mathematics, University of Zagreb, Strossmayerov trg 14, 10000 Zagreb, Croatia

It is known that compounds which contain quinuclidinium and imidazolium subunits possess a wide range of biological activities in the cholinergic system. Some of them were shown to be antidotes against organophosphorus poisoning, as well[1,2].

In this work we describe the synthesis of N-methyl and N-(3-(2-hydroxyiminomethyl-3-methyl-1-imidazolio)-2-oxapropyl derivatives of 3-hydroxy (**I, II**), 3-dimethylcarbamoyloxy (**III, IV**) and 3-acetyloxy (**V, VI**) quinuclidines. Molecular modeling studies were undertaken in order to gain some insight on how different enantiomers of synthesized compounds might interact with acetycholinesterase (AChE) binding sites.



Synthesized quinuclidinium derivatives have been studied as reversible and acylating inhibitors of AChE and protectors against phosphorylation of the enzyme by some warfare agents.

1. Sterling G.H. et al., *Biochem. Pharmacol.* **1993.** *45* 465-472.
2. Simeon-Rudolf V. et al., *Arch. Toxicol.* **1998.** *in press.*

P 53

3-HYDROXYQUINUCLIDINIUM DERIVATIVES: REVERSIBLE AND ACYLATING INHIBITORS OF ACETYLCHOLINESTERASE

E. Reiner, M. Škrinjarić-Špoljar, S. Dunaj, A. Buntić and V. Simeon-Rudolf

Institute for Medical Research and Occupational Health, P.O.Box 291 10001 Zagreb, Croatia

Four compounds were tested for their inhibitory effect on acetylcholinesterase (EC 3.1.1.7): 3-hydroxy-N-methylquinuclidinium iodide (I), N-[3-(3-methyl-2-hydroxyiminomethyl-1-imidazolio)-2-oxa-propyl]-3-hydroxyquinuclidinium dichloride (II) and the dimethyl-carbamates of the compounds I and II (compounds III and IV respectively). Native human erythrocytes were the enzyme source and the activity was measured in 0.1 M phosphate buffer pH = 7.4 at 37 °C with acetylthiocholine (ATCh) as the substrate. The enzyme/inhibitor dissociation constants were evaluated from the apparent dissociation constants as a function of the ATCh concentration (0.05 -1.0 mM). All compounds were reversible inhibitors of AChE and the dissociation constants were between 0.2 and 0.5 mM. Compounds III and IV were also acylating AChE inhibitors; the rate constants of inhibition were 6×10^3 and about 1×10^2 L $mol^{-1}min^{-1}$ for the respective compounds. The oxime group in the compounds II and IV hydrolysed ATCh at a rate of about 20 L $mol^{-1}min^{-1}$ thus interfering with the enzyme catalysed hydrolysis of ATCh. In order to slow down the ATCh/oxime reaction, reversible inhibition by the compounds II and IV was also measured at 10 °C

The compounds were synthesized at the Department of Chemistry, Faculty of Science and Mathematics, University of Zagreb.

P 54

CHEMICAL STRUCTURE - AChE PROTECTIVE ACTIVITY RELATIONSHIP AMONG A SERIES OF BIS-PYRIDINIUM MONO- AND DI-OXIMES

J Lange[1], S Rump[2], T Gidyńska[2], E Gałecka[2], M Kowalczyk[2] and B Serafin[1]
[1]Dept of Technology & Biotechnology of Drugs, Warsaw Technical University and [2]Dept of Pharmacology & Toxicology, Military Institute of Hygiene & Epidemiology, Warsaw, Poland

A series of bis-pyridinium mono- and di-oximes was synthesized and examined for protective activity of acetylcholinesterase (AChE) against organophosphate Soman. Pharmacological experiments were carried out on human erythrocytes and AChE activity was measured after Soman and after the pretreatment with tested compounds according to the method described by Ellman. The structure of compounds was investigated after geometry optimization by molecular mechanics methods. The protective activity of investigated compounds was correlated with the structure, in particular with the position of the amide group and its substituents attached to the 2nd pyridinium ring.

P 55

PECULIARITIES OF INTERACTION OF REVERSIBLE INHIBITORS WITH ACETYLCHOLINESTERASE AND EFFECTIVENESS OF PROPHYLAXIS

V. TONKOPII

Institute for Lake Research, Russian Academy of Sciences;
Sevastyanova str. 9;196199 St. Petersburg, Russia

In order to get some new information about the mechanism of reversible cholinesterase inhibitors (RI) protective action we studied the efficiency of different RI in the experiments *in vitro* and *in vivo*. The following RI were used: galanthamine; tacrine, bis-quaternary compound - ambenonium and some carbamates (physostigmine, aminostigmine and pyridostigmine). The kinetics of the inhibition of the purified human erythrocytes AChE by different RI was studied. It has been found that the competitive RI-galanthamine proved to bind with AChE in the anionic areas of the catalytic centres. The noncompetitive RI-tacrine and ambenonium interacted accordingly with the hydrophobic areas of the enzyme and the allosteric anionic site. Carbamates acted as irreversible inhibitors of AChE - the degree of enzyme inhibition increases with longer incubation time. The effect of RI on phosphorylation of AChE by some OP (armine, soman, Vx) was studied, too. In the presence of galanthamine nnd carbamates, OP interacted only with the free active centres of the enzymes, the active centres of which was not occupied by the RI. Tacrine and ambenonium decreased the reactivity of the free enzyme and the rate of its phosphorylation. In experiments on rats, cats and dogs the preference of competitive RI type of galanthamine and carbamates for prophylaxis against OP poisonings was shown.

P 56

ESTERASES ACTIVITY IN ORGANOPHOSPHORUS POISON PATIENT

Mahmoud Ghazi-Khansari*, Abboss-ali Amoshahie, Mansure Faizie, Mahmoud Rahimie.

Dept of Pharmacology, School of Med, Tehran University of Medical Sciences, P. O. Box 13145-784, TEHRAN, I.R. IRAN (e-mail: Khansagm@nrcgeb.ac.ir)

The widespread use of organophosphorus (OP) insecticides in the control of insects is seriously endangered the human health. OP poisoning is one the most common cause of death due to intentional or accidental poisoning. It is reported that esterases enzyme play an important role in detoxification of OP compounds. In this study, cholinesterase, carboxylesterase and arylesterase enzyme were determined in OP patient before and after treatment. A total of about 55 serum samples from healthy and OP poisoned patient were collected from Khorshid Hospital at Isfahan city and analyzed for esterases activity. The level of cholinesterases, carboxylestreases, and arylesterases activity in OP patient were significantly lower than the healthy person. Further study showed the esterases activity in OP patient were increased significantly after treatment. It is concluded that esterases activity can be used as an indicator of poisoning with OP compounds.

P 57

CATALYTIC MODELS WITH CHOLINESTERASE ACTIVITY

Jiří MATOUŠEK
Faculty of Chemistry, Technical University of Brno
Veslařská 230, CZ-637 00 Brno, Czech Republic

In the research of the catalysts of non-biologic origin, conveniently modelling the esterase activity, we have found that some organic compounds of bifunctional character, having at least one hydroxyl group and in its neighbourhood another group bearing negative charge or at least one free electron pair (separated from the hydroxyl by at least a two-carbon-chain) and activating the hydroxyl group by means of a hydrogen bond formed in an internal chelate complex, strongly accelerate hydrolysis of the substrate - 4-nitrophenyl acetate (NPA).

The catalytic effect is expressed by a substantial drop in the energy of activation of spontaneous hydrolysis of the substrate. The reaction rate of catalysed hydrolysis increases linearly with the concentration of catalyst. The acceleration of the substrate hydrolysis, expressed as the ratio of the reaction rate of the catalysed hydrolysis (k_c) towards the reaction rate of the spontaneous hydrolysis (k_{sp}), i.e. k_c/k_{sp} reaches values up to 50 - 60 in some cases.

The structure-activity relationship of the catalytic models is discussed.

P 58

METHODS FOR THE DETERMINATION OF ORGANOPHOSPHORUS COMPOUNDS BASED ON CHOLINESTERASE INHIBITION USING NON-BIOGENIC CHROMOGENIC AND FLUOROGENIC SUBSTRATES

Jiří MATOUŠEK
Faculty of Chemistry, Technical University of Brno
Veslařská 230, CZ-637 00 Brno, Czech Republic

As compared with the methods for the determination of organophosphorus compounds based on cholinesterase inhibition with biogenic substrates using indirect principles in all cases, the use of the so called chromogenic or fluorogenic substrates offers a possibility of direct determination of the acidic rest of such substrate, splitted in the course of its enzymatic hydrolysis according to the characteristic change of spectral properties or to the fluorescence yield. For the possible use in both simple means of detection, as well as in the automatic alarms, we synthesised and tested several groups of potential chromogenic and fluorogenic substrates - esters of azo dyes, esters of substituted indophenols, esters of substituted nitrophenols and esters of substituted oxyindols and selected the most appropriate compounds from each of these groups.

Spectrophotometric methods using 2,6-dichloroindophenyl acetate and 4-nitrophenylbutyrate are described.

Fluorometric method using indoxylacetate on the fluorometer of own construction - a prototype of field alarm - is described.

P 59

NEW WAYS OF THE DETECTION OF ORGANOPHOSPHORUS COMPOUNDS BASED ON CHOLINESTERASE INHIBITION WITH THIOCHOLINE ESTERS AS SUBSTRATES

Jiří MATOUŠEK
Faculty of Chemistry, Technical University of Brno
Veslařská 230, CZ-637 00 Brno, Czech Republic

Acetylcholine (butyrylcholine) as the biogenic substrate of acetylcholinesterase(and butyrylcholine as substrate of butyrylcholinesterase respectively) were widely used in the first generations of simple means for detection of supertoxic lethal organophosphorus warfare agents and organophosphorus pesticides, such as detection tubes, papers, sheets, as well as in standard methods for determinations used in portable, mobile and stationary laboratories since the 1960s. Because of lesser dependence of the rate of enzymatic hydrolysis on the pH-value, thiocholine esters have been introduced in the second generations of the above mentioned means, the most spread being known method by Ellmann using DTNB as chromogen for visualisation of thiocholine splitted in the course of the enzymatic hydrolysis of substrate. Certain disadvantage of this method especially in the means using the visual coloristic evaluation is the yellow colouration, not very disctinct under limited illumination.

Two instrumental methods, using butyrylthiocholine as substrate, are described, i.e. electrometric method based on electrochemical oxidation of thiocholine measured according to the rate of depolarisation in mV/s, and a spectrophotometric method using as chromogen 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium cloride, specifically prepared (INT^x), yielding a red-violet colouration with an absorption maximum of 500 nm due to corresponding formazan.

P 60

DIMETHYLPHOSPHORUS METABOLITES IN SERUM AND/OR URINE OF PERSONS POISONED BY MALATHION OR THIOMETON

Ž. Vasilić, B. Štengl, V. Drevenkar
Institute for Medical Research and Occupational Health,
Ksaverska c. 2, POB 291, HR -10001 Zagreb, Croatia

The urinary excretion rates of dimethylphosphate, -phosphorothioate and -phosphorodithioate were studied in six persons of whom four had ingested a concentrated solution of malathion and two of thiometon. The concentration decrease of single and total dimethylphosphorus metabolites followed the first-order kinetics with the initial rate constant faster and the later one slower. The excretion rate of total metabolites in the faster phase depended on the their initial concentration in urine. At concentrations higher than 100 nmoles/mg of creatinine the excretion half-times ranged from 7.5 to 15.4 h and at lower concentrations from 40.3 to 55.4 h. Two persons poisoned by malathion were taken blood serum samples for the analysis of the parent pesticide and its metabolites on a daily basis after hospitalization. The parent pesticide was detectable in serum for only one day after the poisoning. The concentration of total malathion dimethylphosphorus metabolites in serum decreased very quickly within 0.5 day after hospitalization. The total metabolite elimination half-times were 4.1 and 4.7 h in the initial faster, and 53.3 and 69.3 days in the later slower elimination phase. There was no correlation between maximum concentrations of total metabolites measured in serum and/or urine on the day of admission to hospital and the initial depression of serum cholinesterase (BChE, EC 3.1.1.8) and erythrocyte acetylcholinesterase (AChE, EC 3.1.1.7).

P 61

ORGANOPHOSPHATE SKIN DECONTAMINATION USING IMMOBILIZED ENZYMES

Richard K. Gordon[1], Shawn R. Feaster[1], David E. Lenz[2], Alan J. Russell[3] and Bhupendra P. Doctor[1].
[1]Walter Reed Army Institute of Research, Washington, DC 20307-5100; [2]U. S. Army Medical Research Institute of Chemical Defense, MD 21010 and [3]Center for Biotechnology and Bioengineering, University of Pittsburgh, PA 15261

We previously demonstrated that a combination of cholinesterase (ChE) pretreatment with an oxime is an effective measure against soman and sarin. We describe here a novel approach for the preparation of covalently linked ChEs which are immobilized to a polyurethane matrix. Such preparation of ChE-sponges enhances the stability and usefulness of the enzymes in non-physiological environments. The ChE-sponges, which can be molded to any form, can effectively be used to remove and decontaminate organophosphates (OPs) from surfaces, biological (skin or wounds) or otherwise (clothing or sensitive medical equipment), or the environment. The ChE-sponges retained their catalytic activity under conditions of temperature, time, and drying where the native soluble enzyme would rapidly denature, and can be reused in conjunction with oximes many times. The ChE-sponge in the presence of oxime repeatedly detoxified OPs such as DFP or MEPQ. These developments in ChE technology have extended the applicability of OP scavengers from *in vivo* protection, to a variety of external detoxification and decontamination schemes. In addition to treatment of OP-contaminated soldiers, the ChE-sponge could protect medical personnel from secondary contamination while attending chemical casualties, and civilians exposed to pesticides or highly toxic nerve agents such as sarin.

P 62

The Role of Carboxylesterase in Development of Resistance to Paraoxon

Wolf D. Dettbarn[1], Zhen P. Yang and Dejan Milatovic
Department of Pharmacology and Neurology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

The contribution of carboxylesterase (CarbE) to the development of tolerance to the organophosphorus anticholinesterase (OP-ANTIChE) paraoxon was investigated in rats. Daily injections (20 days) of paraoxon (0.09 mg/kg) led to a cumulative ED50 dose which was 9.0 fold higher than the acute ED50 dose of 0.20 mg/kg, s.c. During this period, neither visible signs of cholinergic hyperactivity nor death despite persistance of critically reduced brain AChE activity (20-30% of control) were observed. Daily treatment with the CarbE inhibitors CBDP (2-[o-cresyl]-4H-1,2,3-benzodioxa phosphorin-2-oxide) (2 mg/kg, s.c.) or iso-OMPA (tetraisopropylpyro phosphoramide) (3 mg/kg, i.p.) followed by paraoxon (0.09 mg/kg, s.c.) 60 min later prevented the development of tolerance to paraoxon since signs or cholinergic hyperactivity were observed, and rats died on day four of the combined treatment. In tolerant rats one time CBDP or iso-OMPA pretreatment increased toxicity to paraoxon causing death of all rats within 60 min. The increase in paraoxon toxicity was correlated with inhibition of a plasma CarbE, with high affinity toward α-naphtyl acetate (α-NA) and to the inhibitors CBDP, iso-OMPA and paraoxon.

It is concluded that plasma CarbE (α-NA) provided a significant protection against paraoxon intoxication and that the inhibition of this enzyme prevented the tolerance development seen with repeated paraoxon treatments.

[1]Corresponding author. Tel. (615) 936-2592; FAX (615) 936-0223.

P 63

Studies on the degradation of acetylcholinesterase organophosphorus inhibitors using catalytic antibodies

P.Y. Renard[1,2], P. Vayron[1], F. Taran[1], C. Creminon[3], Y. Frobert[3], J. Grassi[3] and C.Mioskowski[1]

1 : SMM/DBCM/DSV, CEA Saclay, F-91191 GIF s/YVETTE Cedex, FRANCE
2 : CE du BOUCHET, BP 3, F-91170 VERT LE PETIT, FRANCE
3 : SPI/DRM/DSV, CEA Saclay, F-91191 GIF s/YVETTE Cedex, FRANCE

The harmful effect of organophosphorus poisonning compounds (insecticides or warfare agents) is often related to their inhibition of mammalian acetylcholinesterase, the enzyme responsible for regulating the *in vivo* concentration of the neurotransmitter acetylcholine. These phosphate or phosphonate esters hydrolysis under physiological conditions has challenged biologists and chemists for many years. To achieve tailored reactions, and to increase the number of chemical reactions available for enzymatic catalysis, mimicry of enzymes mechanisms has been extensively studied. The pioneering work of P. Schultz and R.A. Lerner has confirmed that it is possible to select, from the huge immunoglobulins repertoire, antibodies endowed with catalytic properties for a given reaction. Our studies aimed at the degradation of the exceedingly toxic agent VX (O-ethyl S-(diisopropylaminoethyl) methylphosphonothioate), and of less toxic O-ethyl S-(diisopropylaminoethyl) phenylphosphonothioate with catalytic antibodies. The strategy used for the selection of catalytic antibodies is based on transition state analogs. Haptens bearing an α-hydroxyphosphinate moiety were synthesized, on the ground that such coumponds, which display a high inhibition activity on natural phosphatases, would be stable analogs of the early approach of an hydroxy anion on the phosphorus atom. Polyclonal antibodies have been raised against methylphosphinate, and displayed a noticeable in vitro activity against VX. 13 monoclonal antibodies have been raised against phenylphosphinate, fully characterized via competitive immunoassays (EIA), and their activity against O-ethyl S-(diisopropylaminoethyl) phenylphosphonothioate evaluated.

P 64

Alteromonas Prolidase for Organophosphorus G-agent Decontamination

Tu-chen Cheng[1], Joseph J. DeFrank[1], and Vipin K. Rastogi[2]

Environmental Technology Team, US Army Edgewood Research, Development & Engineering Ctr, Aberdeen Proving Ground, MD 21010, USA[1], Geo-Centers, Inc., Gunpowder Branch, APG, MD 21010, USA[2]
Tel: 410-612-8632; Fax: 410-612-8661; E-mail: tccheng@cbdcom.apgea.army.mil

Organophosphates (OPs) are neurotoxic in many biological systems by virtue of their acetylcholinesterase-inhibitory properties. Enzymes catalyzing the hydrolysis of highly toxic OPs are classified as organophosphorus acid anhydrolase (OPAA; EC 3.1.8.2). Recently, the genes encoding OPAA from two species of *Alteromonas* were cloned and sequenced. Sequence and biochemical analyses of the cloned genes and enzyme has established *Alteromonas* OPAA to be prolidase (E.C. 3.4.13.9), a type of dipeptidase hydrolyzing dipeptides with a prolyl residue in the carboxyl-terminal position (X-Pro). Efforts to over-produce a prolidase from A. *sp*JD6.5 with the goal of developing strategies for long-term storage and decontamination have been successfully achieved. PCR amplified prolidase gene was subcloned into a regulated high-expression pSE420 vector. Following 6 hours IPTG induction, the resulting recombinant cell-line expressed active prolidase up to 50-60% of the total cell protein (~150-200 mg prolidase/L of culture). Large-scale production of this G-agent degrading enzyme, a key pre-requisite for development of a safe and non-corrosive decontamination system, is now feasible with the availability of this recombinant cell line.

Alteromonas prolidase has considerable potential for use in the development of decontamination system for detoxification of a broad range of OPs including G-type chemical nerve agents. The lyophilized enzyme in the presence of trehalose was found to be active and stable for at least one year: Significant activity was retained in $(NH_4)_2C0_2$-reconstituted enzyme solution prepared with different water sources in the presence of biodegradable foams and wetting agents. These findings suggest that *Alteromonas* prolidase has potential use in an enzyme-based and ammonium carbonate-buffered system for variety of sensitive applications such as large area, equipment, and personnel decontamination.

P 65

Degradation of Nerve Gases by CLECs and Cells: Kinetics of Heterogenous Systems

Francis C.G. Hoskin, Marine Biological Laboratory, Woods Hole, MA, USA 02543, and John E. Walker and Robert Stote, U.S. Army Natick RD&E Center, Natick, MA, USA 01760

We have reported the enzymatic hydrolysis of phosphoro- and phosphonofluoridates and phosphoro- and phosphonothiolates and thionates by an organophosphorus hydrolase (OPH) from *Pseudomonas diminuta.* The enzyme has been cross-linked to itself (CLEC = cross-linked enzyme crystals) without a change in the kinetic properties when tested with O,O-diisopropyl S-(2-diisopropylaminoethyl) phosphorothiolate (Tetriso), O,O-diethyl S-(2-ethylthioethyl) phosphorothiolate (Demeton-S), or diisopropyl phosphorofluoridate (DFP) as substrates. When *Pseudomonas diminuta* cells are sonicated, the whole sonicate also hydrolyzes these three compounds in a manner similar to either the purified enzyme or the CLEC-ed enzyme. However, only Demeton-S is hydrolyzed equally well by sonicated cells, purified or CLEC-ed OPH, or whole cells. It appears likely that the protonated form of Tetriso is impeded from crossing the cell membrane in the inward direction, and that the fluoride ion - the detectable product from DFP hydrolysis - is impeded from crossing the cell membrane in the outward direction. Although the CLEC particles are as large as intact *Pseudomonas* cells - indeed, larger - the active sites of the enzyme molecules within the CLEC structure are accessible to the substrate molecule. This is not true of the enzyme molecules within intact cells, these surrounded as they are by a generally lipophilic plasma membrane. Thus in screening for microbial sources of nerve gas hydrolyzing enzymes, Demeton-S has properties which recommend it as a model substrate.

P 66

ORGANOPHOSPHATE INHIBITION OF HUMAN HEART MUSCLE CHOLINESTERASE ISOENZYMES

Jőrg-Michael Chemnitius[a], Rita Sadowski, Holger Winkel, and Ronald Zech. Department of Biochemistry, and Department of Cardiology[a], Georg-August-University, D-37075 Gőttingen, Germany

Acetylcholine hydrolysis of mammalian heart muscle influences cardiac responses to vagal innervation. We characterized cholinesterases of human left ventricular heart muscle in respect to both substrate specificity and irreversible inhibition kinetics with the organophosphorus inhibitor N,N'-diisopropylphosphorodiamidic fluoride (Mipafox). Specimen were obtained postmortem from 3 men and 4 women (61 ± 5 years) with no history of cardiovascular disease. Myocardial choline ester hydrolyzing activity was determined with acetylthiocholine (ASCh; 1.5 mM), acetyl-β-methylthiocholine (AβMSCh; 2.4 mM), and butyrylthiocholine (BSCh; 30 mM). After irreversible and covalent inhibition (60 min; 25°C) with a wide range of mipafox concentrations (50 nM - 5 mM), residual choline ester hydrolyzing activities were fitted to a sum of up to five exponentials using weighted least-squares non-linear curve fitting. In each case, quality of curve fitting reached its optimum on the basis of a four component model. Final classification of heart muscle cholinesterases was achieved according to substrate hydrolysis patterns and second-order mipafox inhibition rate constants (k2):

	Mipafox inhibition rate constant	**Substrate hydrolysis** (nmol/min/g w.w.)		
Isoenzymes	k_2 (l/mol/min)	ASCh	AβMSCh	BSCh
Acetylcholinesterase, AChE	6.1 (± 0.8) x 10^2	115 ± 18	77 ± 9	9 ± 3
Cholinesterase, ChE	5.3 (± 1.1) x 10^3	59 ± 13	38 ± 8	64 ± 14
Atypical Cholinesterase	5.2 (± 1.0) x 10^{-1}	9 ± 3	10 ± 2	39 ± 4
Cholinesterase II	6.4 (± 2.0) x 10^4	40 ± 6	22 ± 5	101 ± 18

One choline ester hydrolyzing enzyme was identified as acetylcholineesterase (AChE; EC 3.1.1.7), and one as cholinesterase (ChE; EC 3.1.1.8). An enzyme exhibiting both ChE-like substrate specificity and relative resistance to mipafox inhibition was classified as atypical cholinesterase. ChE II, according to mipafox inhibition and substrate hydrolysis, probably is of nonmyocardial origin.

P 67

DICHLOROPHENYL PHOSPHORAMIDATES AS SUBSTRATES FOR PHOSPHOTRIESTERASES IN AVIAN AND OTHER SPECIES: variability in level, calcium dependence and stereoespecificity.

A. Monroy, M.A. Sogorb, N. Díaz-Alejo, J.L.Vicedo, J. Barril, E. Vilanova, University Miguel Hernandez. Unit of Toxicology and Chemical Safety. Campus de San Juan (Alicante). Spain.

Using paraoxon as substrate, phosphotriesterases have been considered absent in avian tissues. However chicken and other species contain activities hydrolysing dichlorophenyl phosphoramidates (HDCPase) in serum and liver and brain. A non calcium dependent and non stereoespecific activity are associated to serum albumin in all species that seems to be mediated by a phosphorylated intermediate similar to a B-esterase mechanism with low turnover number but toxicologically relevant due to the high concentration. In tissues (including serum lipoprotein fraction, soluble and membrane fractions of liver and brain) of several tested species (chicken, rat, rabbit) and human serum, there are calcium depending activities varying in level and stereoespecificity from cases undetectable level in chicken serum to veiy high an stereoespecific activity in rabbit serum lipoproteins, being human intermediate level and non stereoespecific. In many tissues and purified preparations, the paraoxonase level is lower that the HDCPase activity. It could explain why avian was considered lacking phosphotriesterase activity, as paraoxon is the usual substrate suggesting that probably HDCP could be a more effective and sensitive substrate than paraoxon for measuring phosphorotriesterase activity. Work supported by CICYT grant SAF96/168

P 68

EFFECTS OF ORGANOPHOSPHATES ON CHOLINESTERASE ACTIVITY AND NEURITE REGENERATION IN *APLYSIA*

M. Srivatsan, Department of Physiology, University of Kentucky College of Medicine, Lexington, KY 40536-0084, U.S.A.

Acetylcholinesterase (AChE) which hydrolyzes acetylcholine (ACh) at the cholinergic synapses is present in cholinergic and noncholinergic neurons and in hemolymph *of Aplysia,* a marine mollusc. *Aplysia* hemolymph has a very high level of AChE which promotes neurite growth in primary cultures of dopaminergic neurons via a non-cholinergic mechanism (Srivatsan and Peretz, 1997). In contrast, AChE is reported to facilitate neurite growth in cholinoceptive neurons by hydrolyzing ACh which inhibits neurite growth (Small et al., 1995). Hence we investigated the effects of active-site inhibited hemolymph AChE on neurite growth of cholinergic neurons of *Aplysia* in primary culture. Organophosphates being long-acting active site inhibitors of AChE were chosen for this study. The effects of active site inhibited hemolymph AChE was tested on large cholinergic neurons, R2 (abdominal ganglion) and LPL1 (left pleural ganglion) as well as small cholinergic neurons (buccal ganglion) of *Aplysia,* maintained in culture. Hemolymph AChE was inhibited by preincubation with either 10 μM of echothiophate (I50 = 0.74 μm) or 5 μM of paraoxon (I50 = 0.02 μM). Neurons were maintained in (1) L15 (culture medium) alone, (2) L15 + hemolymph AChE and (3) L15 + inhibited hemolymph AChE. Neuron viability and neurite growth were examined in these neurons after 12, 24 and 48 hours in culture. Addition of uninhibited hemolymph AChE enhanced the survival and significantly increased neurite growth of cultured neurons compared to L15 alone. In the presence of echothiophate- inhibited AChE, neuronal survival was not affected; however neurite growth was significantly reduced when compared to L15 + uninhibited AChE. The presence of paraoxon -inhibited AChE in the medium impaired both survival and neurite growth in the cultured neurons. Thus the effect of paraoxon on cholinegic neurons of *Aplysia* appears to differ from that of echothiophate.

P 69

PURIFICATION AND KINETICS OF GUINEA-PIG ESTERASES HYDROLYZING ESTERIFIED MONOSACCHARIDES

V. Petrović-Peroković, J. Tomašić*, Z. Mihalić and S. Tomić

Department ot Chemistry, Faculty of Science and Mathematics, University of Zagreb, Strossmayerov trg 14,10000 Zagreb, Croatia

**Institute of Immunology Inc., Rockefellerova 2, 10000 Zagreb, Croatia*

Mammalian serum is known to be a rich source of different enzymes, some of which can be used as catalysts in selective transformations of sugars. It was also shown that rabbit[1] and guinea pig[2] sera contain some esterases specific for acylated monosaccharides, but the regioselectivity of hydrolysis differs depending on the enzyme source.

In this work we report on further purification, catalytic and kinetic properties of two different esterases isolated from guinea-pig serum showing various degrees of activity and different regioselectivities.

[1]S. Tomić, A. Treščec, Đ. Ljevaković, J. Tomašić, *Carbohydr. Res.***1991**, 210,191.
[2]S. Tomić, M. Krstanović and J. Tomašić, *Period biol* **1996**, 98(3), 337.

P 70

INHIBITION OF THE ACID PHOSPHATASE IN THE SEEDS OF *Pulsatilla grandis* WITH ZEOLITE A

Leon Senčič and Boštjan Štih

Department of Biology, Pedagogical Faculty, University of Maribor, Koroška c. 160, 2000 Maribor, Slovenia.

Zelite A inhibits germination and the acid phosphatase activity of the seeds of *Pulsatilla grandis.* The effective concentration of 50% inhibition (EC_{50}) of acid phosphatase *in vivo* was 11.5 g/L. The partial purifed enzyme was preliminary assessed to be a neutral molecule with a molecular weight no less than 80 000. The K_m was determined 0.1 mol/L by using the 4-nitrophenyl phosphate as a substrate. Freezing and thawing decreased the activity and at constant pH (in citrate buffer) the effect of zeolite A up to 100 g/L was not detected, indicating that the mechanism of inhibition with zeolite A is a consequence of elevating the pH.

P 71

ISOESTERASES RELATED TO CELL DIFFERENTIATION IN PLANT TISSUE CULTURE

Marijana Krsnik-Rasol, Hana Čipčić
Faculty of Science and Mathematics, Department of Biology, Rooseveltov trg 6, 10000 Zagreb, Croatia

In vitro culture of cells and tissues provides a powerful way to study developmental processes in plants. Biochemical markers may be useful in predicting developmental events which are caused by modifications of gene-expression program. Isoesterases were separated electrophoretically in gradient (8-15%) gels of polyacrylamide and by isoelctric focussing in a wide pH (3-10) range, using naphtylacetate as a substrate. Differentially specific patterns were obtained. Transformation of potato (*Solanum tuberosum* L.) tuber cells with *Agrobacterium tumefaciens* and crown gall tumour growth were characterised by changes in isoesterases pattern. A new isoenzyme, probably related to stress in the culture conditions, appeared in both the control and tumour tissue. Tumour cells stopped producing at least two isoenzymes. In horse radish (*Armoracia lapathifolia* Gilib.) tumour culture two cathodic isoenzymes were distinct for teratomic tissue. Mainly quantitative differences in isoesterases pattern were noticed in normal, habituated and transformed tissue lines of sugar beet (*Beta vulgais* L.). As the results of isoelectric focussing showed, tumour tissue produced less isoenzymes than the other tissue lines. The obtained results confirmed esterases as reliable marker enzymes in cell differentiation, but further investigation should reveal their biological function.

P 72

MOLECULAR CHARACTERIZATION OF PHOSPHODIESTERASES IN HUMAN CORPUS CAVERNOSUM

Andrea Küthe, Heike Eckel, Hans-J. Mägert, Christian G. Stief, Stefan Ückert, Wolf-G. Forssmann, Udo Jonas, Hannover Medical School, Department of Urology and Institute for Peptide Research, Hannover, Germany

Cyclic nucleotide phosphodiesterases hydrolyse cyclic AMP and cyclic GMP which are known to be intracellular second messengers mediating human cavernous smooth muscle relaxation. Molecular characterization of these enzymes may give supplementary view into the physiology of erection and pathophysiology of erectile dysfunction. In the past we have shown (J. Urol. 149: 285 (A), 1993) the existence of three different phosphodiesterase isoenzymes in human cavernous tissue: PDE III (cGMP-inhibited cAMP-phosphodiesterase), PDE IV (cAMP-specific phosphodiesterase) and PDE V (cGMP-specific phosphodiesterase). Functional organ bath studies (J. Urol. 151: 495 (A), 1994) demonstrated the importance of PDE III in inducing cavernous smooth muscle relaxation in. vitro. Therefore, molecular biological analyses comparing cavernous tissue from patients with and without erectile dysfunction and various urogenital organs were performed in order to get information concerning the expression and to determine the complementary deoxyribonucleic acid (cDNA) sequence of PDE III.

Human corpus cavernosum tissue was obtained from patients who underwent implantation of penile prothesis and correction of penile deviation. Total ribonucleic acid (RNA) was isolated and subjected to RT-PCR analysis using primers derived from the sequences data of the human myocardial PDE III and human PDE V. Nucleotide sequence were determined by means of an automatic flourescence sequencer.

PDE III shows the highest expression level in corpus cavernosum followed by PDE V compared with other human urogenital tissues. The expression pattern of PDE III in cavernous tissue from potent and impotent patients shows no quantitative difference. cDNA sequence from myocardial and cavernous tissue show 100% homology. However, they are different in some nucleotides compared with the published human myocardial PDE III (GenBank).

Although we know PDE III in corpus cavernosum is of functional relevance in the regulation of cavernous smooth muscle tone, our data indicate that altered nucleotide sequence of cavernous PDE III is not a cause for erectile dysfunction. Further investigations on the identification of upstream regulatory factors being involved in the development of erectile dysfunction are necessary.

Supported by a grant from the DFG (STI 96/10-1) and from Gesellschaft der Freunde der Medizinischen Hochschule Hannover.

P 73

Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides

François Villatte, Véronique Marcel, Sandino Estrada-Mondaca and Didier Fournier

Laboratoire d'entomologie appliquée, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France
E-mail: fournier@cict.fr; fax: 33.5.61.55.69.10

Several systems using acetylcholinesterase have been developped to detect carbamate and organophosphate insecticides from food or water samples. We have studied enzyme from bovine erythrocyte, Electrophorus electricus, Drosophila melanogaster, Torpedo californica and Caenorhabditis elegans, to determine the best adapted enzyme to use in biosensors. It appeared that insect acetylcholinesterase was more susceptible to a broad range of organophosphates and carbamates insecticides than the other tested enzymes.
In addition, this enzyme have been rendered 12-fold more sensitive to some insecticides by in vitro mutagenesis of Y408(330), compared to the Electrophorus electricus enzyme.

Third International Meeting on ESTERASES REACTING WITH ORGANOPHOSPHORUS COMPOUNDS

Late submitted poster abstracts

SERUM LDH ACTIVITY AND LDH ISOENZYME CHANGE FOLLOWING SHORT-TERM EXPOSURE TO METHOMYL IN RATS AND LDH ISOENZYME CHANGE IN POISONED SPRAYMEN

Parlap Sinhaseni and Ornrat Lohitnavy

Institute of Health Research, Chulalongkorn University, SOI Chulalongkorn 62, Phyathay Rd., Patumwan, Bangkok 10330, Thailand

The toxicities of methomyl by a single and repeated administration were investigated in rats. Rats treated with single doses of 3-7 mg/kg of methomyl showed significant increase in total lactate dehydrogenase (LDH) activity on day 1 and the highest activity was observed in rats receiving 7 mg/kg of methomyl on day 3.
Subsequently, the total LDH activity declined to normal level on day 7 after dosing. Concurrently, specific increase in LDH-3 and -4 isoenzyme levels were observed. On the other hand, both spleen weight and splenocyte viability were significantly reduced in rats treated with acute dose of 6 and 8 mg/kg of methomyl on days 1 and 3, respectively. However, splenotoxicity was protected by pretreatment of rats with N-acetylcisteine (NAC). From these results, it is likely that splenotoxicity of methomyl may be due, at least in part, to free radical formation.
Lactate dehydrogenase isoenzyme profile change was also observed in 2 cases of methomyl poisoned spraymen.

ACID PHOSPHATASE FROM WHEAT GERMS IN PHOSPHOENOLPYRUVATE HYDROLYSIS ACTS IN ASSOCIATIVE FORM AS PYROPHOSPHATE-DEPENDENT ENZYME

Natalia Samus, Alexander Yasnikov

Institute of bioorganic chemistry and petrochemistry, Ukr. Nat. Acad. Sci. Murmanskaya, 1, Kiev, 253660, UKRAINE. E-mail: samus@bpci.kiev.ua

Phosphoenolpyruvate (PEP) hydrolysis by acid phosphatase (Apase) from weat germs was studied in comparison with *p*-nitrophenylphosphate (NPP), ATP and ADP. It was shown that two molecules of Apase involve in this reaction. At the same time NPP, ATP, ADP and are hydrolysed on the each subunit of Apase. Pyrophosphate (Ppi) has catalytic effect on the PEP hydrolysis. During the reaction phosphorylation of the Apase active site occurs and Ppi, which proves to be an allosteric regulator, is formed. After the comparison of enzymatic and biomimetic catalysis such mechanism is considered: PEP double link is activated by the formation of complex with Apase proton-donor group and than transfer of PEP phosphoryl residue onto the imidasol group of the enzyme occurs.

AUTHOR INDEX

H

J

K

L

M

LIST OF PARTICIPANTS

Gabriel AMITAI
Department of Pharmacology
Israel Institute for Biological Research
24 Reuven St., P.O. Box 19
74100 Ness-Ziona, **Israel**
Tel: (972-8) 9381591
Fax: (972-8) 9381559
E-mail: amitai@iibr.gov.il

Yacov ASHANI
Israel Institute for Biological Research
24 Reuven St., P.O. Box 19
74100 Ness-Ziona, **Israel**
Tel: (972-8) 9381455
Fax: (972-8) 9401094
E-mail: yashani@netvision.net.il

Dov BARAK
Department of Organic Chemistry
Israel Institute for Biological Research
24 Reuven St., P.O. Box 19
74100 Ness-Ziona, **Israel**
Tel: (972-8) 9381529
Fax: (972-8) 9401094
E-mail: barak@iibr.gov.il

Jose BARILL
Unidad de Toxicología
Universidad Miguel Hernandez
Campus de San Juan
03550 San Juan (Alicante), **Spain**
Fax: (34-6) 5919484

Paul BEILSTEIN
Novartis Crop Protection AG
R-1058.7.22, Postfach
4002 Basel, **Switzerland**
Tel: (41-61) 6978320
Fax: (41-61) 6974966
E-mail: paul.beilstein@cp.novartis.com

Scott Stephen BILLECKE
University of Michigan, Anesthesiology
Room 7433 Medical Science 1 Bldg.
1150 West Medical Center Drive
Ann Arbor, MI 48109-0615, **USA**
Tel: (1-734) 7645197
Fax: (1-734) 7649330
E-mail: bilker@umich.edu

Clarence A. BROOMFIELD
Biochemical Pharmacology Branch
US Army Medical Research Institute of
Chemical Defence
3100 Ricketts Point Road
APG, MD 21010-5425, **USA**
Tel: (1-410) 6712626
Fax: (1-410) 6711960
E-mail: broomfield@asia.apgea.army.mil

Arnaud CHATONNET
Departement de Physiologie Animale
INRA - DCC
2, place P. Viala
34060 Montpellier cedex 1, **France**
Tel: (33-4) 99612677
Fax: (33-4) 67545694
E-mail: chatonne@ensam.inra.fr

Jörg-Michael CHEMNITIUS
Department of Cardiology
Center of Internal Medicine
Georg-August-Universität
Robert-Koch-Strasse 40
37075 Göttingen, **Germany**
Tel: (49-551) 396310
Fax: (49-551) 392026

Tu-Chen CHENG
US Army ERDEC
SCBRTL-RTL, E3220
APG, MD 21010-5423, **USA**
E-mail: tccheng@CBDCOM-EMH1.APGEA
.ARMY.MIL

Cecile CLERY
CRSSA - unite de biochimie, BP 87
38702 La Tronche cedex, **France**
Tel: (33-4) 76636989
Fax: (33-4) 76636961
E-mail: 101511.3606@compuserve.com

Ephraim COHEN
Department of Entomology
Faculty of Agriculture
The Hebrew University of Jerusalem
76100 Rehovot, **Israel**
Tel: (972-8) 9481118
Fax: (972-8) 9466768
E-mail: ecohen@agri.huji.ac.il.

Lourdes Rodrigo CONDE-SALAZAR
Dept of Legal Medicine and Toxicology
Faculty of Medicine
University of Granada
Avda. Madrid, 11
18071 Granada, **Spain**
Tel: (34-58) 243546
Fax: (34-58) 243491

Lucio G. COSTA
Department of Environmental Health
4225 Roosevelt Way, NE, Suite #100
Seattle, WA 98105-6099, **USA**
Tel: (1-206) 5432831
Fax: (1-206) 6854696
E-mail: lcosta@dehpost.sphcm.washington.edu

Wolf D. DETTBARN
Department of Neurology
Vanderbilt University
Medical Center South
2100 Pierce Avenue
Nashville, TN 37212-3375, **USA**
Tel: (1-615) 9360060
Fax: (1-615) 9360223
E-mail: dettbawd@ctrvax.vanderbilt.edu

Christophor DISHOVSKY
Department of Toxicology
Institute for Physiology
Bulgarian Academy of Science
A. Bonchev Bl. 23
1113 Sofia, **Bulgaria**
Tel: (359-2) 7132107
Fax: (359-2) 738469
E-mail: chdishov@iph.lio.acad.bg

Bhupendra P. DOCTOR
Division of Biochemistry
Walter Reed Army Institute of Research
Washington, D.C. 20307-5100, **USA**
Tel: (1-202) 7823001
Fax: (1-202) 7826304
E-mail: Dr_Buphendra_Doctor@wrsmtp-ccmail.army.mil

Jonathan Alan DOORN
c/o Dr. Bert La Du
University of Michigan, Anesthesiology
Room 7433 Medical Science 1 Bldg.
1150 West Medical Center Drive
Ann Arbor, MI 48109-0615, **USA**
Tel: (1-734) 7645197
Fax: (1-734) 7649332
E-mail: jdoorn@umich.edu

Vlasta DREVENKAR
Institute for Medical Research and
Occupational Health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398
E-mail: vdreven@mimi.imi.hr

Shawn Ray FEASTER
Division of Biochemistry
Walter Reed Army Institute of Research
Washington D.C. 20307, **USA**
Tel: (1-202) 7827653
Fax: (1-202) 7826327
E-mail: Dr._Shawn_Feaster@wrsmtp-ccmail.army.mil

Zlata FLEGAR-MEŠTRIĆ
Institute of Clinical Chemistry
Clinical Hospital "MERKUR"
Zajčeva 19
10000 Zagreb, **Croatia**
Tel: (385-1) 212220
Fax: (385-1) 212220

Didier FOURNIER
Laboratoire d' Entomologie
Université Paul Sabatier
31062 Toulouse, **France**
Fax: (33-05) 61556910
E-mail: fournier@cict.fr

Clement FURLONG
Department of Genetics, SK-50
School of Public Health and
Community Medicine
University of Washington
Seattle, WA 98195-7360, **USA**
Tel: (1-206) 5431193
Fax: (1-206) 5430754

Mahmoud GHAZI-KHANSARI
Department of Pharmacology
School of Medicine
Tehran University of Medical Sciences
P.O. Box 13145-784
Tehran, **Iran**
Tel: (98-21) 6402569
Fax: (98-21) 6057519
E-mail: khansagm@nrcgeb.ac.ir

Fernando GIL HERNANDEZ
Department of Legal Medicine and
Toxicology
Faculty of Medicine
University of Granada
Avda. Madrid, 11
18071 Granada, **Spain**
Tel: (34-58) 243546
Fax: (34-58) 243491
E-mail: fgil@goliat.ugr.es

Paul GLYNN
MRC Toxicology Unit
Hodgkin Building
University of Leicester , P.O. Box 138
Lancaster Road
Leicester LE1 9HN, **United Kingdom**
Tel: (44-116) 2525598
Fax: (44-116) 2525616
E-mail: pg8@le.ac.uk

Maurice Ph. GOELDNER
Laboratoire de Chimie Bio-organique
URA 1386 CNRS
Faculté de Pharmacie
ULP-Strasbourg 1, BP 24
67401 Illkirch Graffenstaden, **France**
Tel: (33-3) 88676991
Fax: (33-3) 88678891
E-mail: goeldner@pharma.u-strasbg.fr

Azra GRANOV
Quality Assurance Division Manager
SANOFARM
Jukićeva 53
71000 Sarajevo, **Bosna i Hercegovina**
Tel: (387-71) 208570 or 663955
Fax: (387-71) 470352

Zoran GRUBIČ
Institute for Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1001 Ljubljana, **Slovenia**
Tel: (386-61) 310841
Fax: (386-61) 302272
E-mail: grubic@ibmi.mf.uni-lj.si

Rajendra GUPTA
Department of Botany
University of Delhi
110007 Delhi, **India**
Tel: (91-11) 7256175
Fax: (91-11) 7256541
E-mail: rajendra@csec.ernet.in

Brennie E. HACKLEY
US Army Medical Research Institute of Chemical Defence
3100 Ricketts Point Road
APG, MD 21010-5425, **USA**
Tel: (1-410) 6713276
Fax: (1-410) 6714150
E-mail: dr._brennie_hackley@ftdetrck-ccmail.army.mil

Wolfgang HARDEGG
Medical Faculty
University of Heidelberg
Im Gabelacker 3
69120 Heidelberg, **Germany**
Tel: (49 6221) 412356
Fax: (49 6221) 565690

Ursula HELFRICH
Dept of Pharmacology and Toxicology
University of Heidelberg at Mannheim
Maybach Street 14-16
68169 Mannheim, **Germany**
Tel: (49-621) 331067
Fax: (49-621) 377601

Snježana HERCEG
Institute for Medical research and Occupational Health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398

Antonio F. HERNANDEZ JEREZ
Department of Legal Medicine and Toxicology
Faculty of Medicine
University of Granada
Avda. Madrid, 11
18071 Granada, **Spain**
Tel: (34-58) 243546
Fax: (34-58) 243491
E-mail: ajerez@goliat.ugr.es

Eberhard HEYMANN
Physiological Chemistry
University of Osnabrück
49069 Osnabrück, **Germany**
Tel: (49-541) 9692463 or 9692467
Fax: (49-541) 9692444

Francis C.G. HOSKIN
c/o Dr. John E. Walker
US Army Natick RD&E Center
Natick, MA 01760-5020, **USA**
Tel: (1-508) 2334472
Fax: (1-508) 2335521
E-mail: jowalker@natick-amed02.army.mil

Lakshmanan JAGANATHAN
Department of Biotechnology
Bharathiar University
641046 Coimbatore, **India**
E-mail: biotech@as250.bharathi.ernet.in

Martin K. JOHNSON
M.R.C. Toxicology Unit
Hodgkin Building
University of Leicester, P.O. Box 138
Lancaster Road
Leicester LE1 9HN, **United Kingdom**
Tel: (44-116) 2525585
Fax: (44-116) 2525616

Denis JOSSE
Eppley Institute
University of Nebraska Medical Center
600 S. 42nd St., P.O. Box 986 805
Omaha, NE 68198-6805, **USA**
Tel: (1-402) 5596014
E-mail: djosse@mail.unmc.edu

Ivan JUKIĆ
Croatian Military Academy
Ministry of Defence of the Republic of Croatia
Ilica 256 b
10000 Zagreb, **Croatia**
Tel: (385-1) 3786388
Fax: (34-58) 3786149

Wolfgang JUNGE
Zentrallaboratorium
Friedrich-Ebert Krankenhaus
Friesenstrasse 11
24531 Neumünster, **Germany**
Tel: (49-4321) 4053811
Fax: (49-4321) 4053809

Daniella KAUFER
Department of Biological Chemistry
Institute of Life Sciences
The Hebrew University of Jerusalem
91904 Jerusalem, **Israel**
Tel: (972-2) 6585454
Fax: (872-2) 6520258
E-mail: soreq@shum.huji.ac.il

Anton KLINK
Medizinischer Berater
GTI Industrievertretungen
53229 Bonn, **Germany**
Tel: (49-228) 9481357
Fax: (49-228) 9481359
E-mail: BERECH@compuserve.com

Zrinka KOVARIK
Institute for Medical Research and
Occupational Health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398
E-mail: zkovarik@mimi.imi.hr

Marek KOWALCZYK
Department of Pharmacology and Toxicology
Military Institute of Hygiene and
Epidemiology
Kozielska 4
01-163 Warszawa, **Poland**
Tel: (48-22) 6108459
Fax: (48-22) 104391

Marijana KRSNIK-RASOL
Department of Biology
Faculty of Science and Mathematics
Rooseveltov trg 6
10000 Zagreb, **Croatia**
Tel: (1-385) 4826261
Fax: (1-385) 4826260
E-mail: mkrsnik@public.srce.hr

Andrej KUNSTELJ
Institute of Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1000 Ljubljana, **Slovenia**
Tel: (386-61) 310841
Fax: (386-61) 302272

Andrea KÜTHE
Institute for Peptide Research
Feodor-Lynen-Strasse 31
30625 Hannover, **Germany**
Tel: (49-511) 5466322
Fax: (49-511) 5466102

Bert N. LA DU
University of Michigan, Anesthesiology
Room 7433 Medical Sciences 1 Bldg.
1150 West Medical Center Drive
Ann Arbor, MI 48109-0615, **USA**
Tel: (1-734) 7636429
Fax: (1-734) 7649332
E-mail: bladu@umich.edu

Haim N. LEADER
Department of Pharmacology
Israel Institute for Biological Research
24 Reuven St., P.O. Box 19
74100 Ness-Ziona, **Israel**
Tel: (972-8) 9381551
Fax: (972-3) 9663819
E-mail: leader@iibr.gov.il

Oksana LOCKRIDGE
Eppley Institute
University of Nebraska Medical Center
600 S. 42nd St., P.O. Box 986805
Omaha, NE 68198-6805, **USA**
Tel: (1-402) 5596032
Fax: (1-402) 5594651
E-mail: olockrid@mail.unmc.edu

Marcello LOTTI
Instituto di Medicina del Lavoro
Universita degli Studi di Padova
via Giustiniani 2
35128 Padova, **Italy**
Tel: (39-49) 8212548/8212549
Fax: (39-49) 8212550
E-mail: lottitox@ux1.unipd.it

Ana LUCIĆ
Institute for Medical Research and
Occupational Health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398

Chunyuan LUO
Division of Biochemistry
Walter Reed Army Institute of Research
6825 16th St. NW
Washington, D.C. 20307-5100, **USA**
Tel: (1-202) 7827245
Fax: (1-202) 7827651
E-mail: dr. chunyuan luo@wrsmtp-ccmail.army.mil

Bharti MACKNESS
Department of Medicine
Manchester Royal Infirmary
Oxford Road
Manchester MI3 9WL, **United Kingdom**
Tel: (44-161) 2768633
Fax: (44-161) 2744833

Michael MACKNESS
Department of Medicine
Manchester Royal Infirmary
Oxford Road
Manchester M13 9WL, **United Kingdom**
Tel: (44-161) 2768633
Fax: (44-161) 2744833
E-mail: mmack@central.cmht.nwest.nhs.uk

Galina F. MAKHAEVA
Department of Pharmacology
Institute of Physiologically Active Substances
Russian Academy of Sciences
Chernogolovka
Moscow Region 142432, **Russia**
Tel: (7-095) 5245062
Fax: (7-095) 9628061
E-mail: makh@pharm.unicon.msk.su

Vladimir V. MALYGIN
Department of Pharmacology
Institute of Physiologically Active Substances
Russian Academy of Science
Chernogolovka
Moscow Region 142432, **Russia**
Tel: (7-095) 9628080 ext 44-586
Fax: (7-095) 9628061
E-mail: makh@pharm.unicon.msk.su

Eugeni Dimitrova MANOLOVA
Laboratory of Experimental Toxicology
Military Medical Academy
St. G. Sofiiski Str. 3
1606 Sofia, **Bulgaria**
Tel: (359-2) 51542472

Pascale MARCHOT
CNRS UMR 6560 Ingénierie des Protéines
Institut Fédératif de Recherche Jean Roche
Faculté de Médecine Secteur Nord
Boulevard Pierre Dramard
13916 Marseille cedex 20, **France**
Tel: (33-4) 91698714
Fax: (33-4) 91657595
E-mail: marchot.p@jean-roche.univ-mrs.fr

Patrick MASSON
Department of Toxicology
Biochemistry Unit
CRSSA, P.O. Box 87
24, avenue des Maquis du Grésivaudan
38702 La Tronche cédex, **France**
Tel: (33-476) 636959
Fax: (33-476) 636901
E-mail: 100335.404@compuserve.com

Jean MASSOULIÉ
Laboratoire de Neurobiologie
Ecole Normale Superieure
CRNC-URA 295
46 rue d'Ulm
F-75005 Paris, **France**
Tel: (33-1) 44323891
Fax: (33-1) 44323887
E-mail: massouli@biologie.ens.fr

Laszlo MATE
Ministry of Foreign Affairs
Galgoczy u. 50. B
1125 Budapest, **Hungary**
Tel: (361) 2142829
Fax: (361) 2142829
E-mail: calvin@terminus.inext.hu

Jiří MATOUŠEK
Institute for Chemistry and Technology of Environmental Protection
Faculty of Chemistry
Technical University of Brno
Veslařská 230
637 00 Brno, **Czech Republic**
Tel: (420-5) 43321295
Fax: (420-5) 43211101
E-mail: matousek@fch.vutbr.cz

Donald M. MAXWELL
Biochemical Pharmacology Branch
US Army Medical Research Institute of Chemical Defence
3100 Ricketts Point Road
APG, MD 21010-5425, **USA**
Tel: (1-410) 6711315
Fax: (1-410) 6711960

Charlene M. MELLO
Biotechnology
US Army Natick RD & E Center
Kansas St.
Natick MA 01760-5020, **USA**
Tel: (1-508) 2335825
Fax: (1-508) 2335521

Tanja MILANIČ
Institute of Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1000 Ljubljana, **Slovenia**
Tel: (386-61) 310841
Fax: (386-61) 302272

Sanja MILKOVIĆ-KRAUS
Institute for Medical Research and Occupational Health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 420412
Fax: (385-1) 420398
E-mail: skraus@mimi.imi.hr

Charles B. MILLARD
Department of Neurobiology
The Weizmann Institute of Science
76100 Rehovot, **Israel**
Tel: (972-8) 9342128
Fax: (972-8) 9344131
E-mail: limill@weizmann.weizmann.ac.il

Katarina MIŠ
Institute of Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1000 Ljubljana, **Slovenia**
Tel: (386- 61) 310841
Fax: (386-61) 302272

Antonio MONROY
Unidad de Toxicología
Universidad Miguel Hernandez
Campus de San Juan
03550 San Juan (Alicante), **Spain**
Fax: (34-6) 5919484

David H. MOORE
Deputy Commander
US Army Medical Research Institute of Chemical Defence
3100 Ricketts Point Road
APG, MD 21010-5425, **USA**
Tel: (1-410) 6713278
Fax: (1-410) 6714150
E-mail: col_david_moore@ftdetrck-ccmail.army.mil

Angelo MORETTO
Instituto di Medicina del Lavoro
Universita degli Studi di Padova
via Giustiniani 2
35128 Padova, **Italy**
Tel: (39-49) 8212548/8212549
Fax: (39-49) 8212550

Wendy Anne ODGERS
Biotechnology Section
CSIRO Entomology
PO Box 1700, ACT, 2601, **Australia**
Tel: (61-6) 2464159
Fax: (61-6) 2464173
E-mail: wendyo@ento.csiro.au

Renata ODŽAK
Department of Chemistry
Faculty of Science and Mathemathics
University of Zagreb
Strossmayerov trg 14
10000 Zagreb, **Croatia**
Tel: (385-1) 4819289
Fax: (385-1) 4819288
E-mail: roncevic@olimp.irb.hr

Arie ORDENTLICH
Department of Biochemistry
Israel Institute for Biological Research
24 Reuven St., PO Box 19
74100 Ness-Ziona, **Israel**
Tel: (972-8) 9381531
Fax: (972-8) 9401094
E-mail: barak@iibr.gov.il

Georg PETROIANU
Department of Pharmacology
University of Heidelberg at Mannheim
Maybach St. 14-16
68169 Mannheim, **Germany**
Tel: (49-621) 331064
Fax: (49-621) 377601

Iskra Iordanova PETROVA
Laboratory of Experimental Toxicology
Military Medical Academy
St. G. Sofiiski Str. 3
1606 Sofia, **Bulgaria**
Tel: (359-2) 51542472

Vesna PETROVIĆ-PEROKOVIĆ
Department of Chemistry
Faculty of Science and Mathematics
Sveučilište u Zagrebu
Strossmayerov trg 14
10000 Zagreb, **Croatia**
Tel: (385-1) 4819289
Fax: (385-1) 4819288
E-mail: vpetrovic@olimp.irb.hr

Antonio PLA MARTINEZ
Department of Legal Medicine and Toxicology
Faculty of Medicine
University of Granada
Avda, Madrid 11
18071 Granada, **Spain**
Tel: (34-58) 243546
Fax: (34-58) 243491/234401
E-mail: apla@goliat.ugr.es

Peter L. PLATTEBORZE
Biochemical Pharmacology Branch
US Army Medical Research Institute of
Chemical Defence
3100 Ricketts Point Road
APG, MD 21010-5425, **USA**
Tel: (1-410) 6711727
Fax: (1-410) 6711960
E-mail: cpt_peter_platteborze@ftdetrck-ccmail.army.mil

Radovan PLEŠTINA
Bukovačka cesta 269
10000 Zagreb, **Croatia**
Tel: (385-1) 243289

Ines PRIMOŽIČ
Deparment of Chemistry
Faculty of Science and Mathemathics
University of Zagreb
Strossmayerov trg 14
10000 Zagreb, **Croatia**
Tel: (385-1) 4819289
Fax: (385-1)4819288
E-mail: primozic@olimp.irb.hr

Daniel M. QUINN
Department of Chemistry
The University of Iowa
Iowa City, IA 52242, **USA**
Tel: (1-319) 3351335
Fax: (1-319) 3351270
E-mail: daniel-quinn@uiowa.edu

Zoran RADIĆ
Department of Pharmacology
University of California San Diego
School of Medicine
9500 Gilman Drive, Dept. 0636
La Jolla, CA 92093-0636, **USA**
Tel: (1-619) 5344026
Fax: (1-619) 5346833
E-mail: zoran@phrtayl0.ucsd.edu

Frank M. RAUSHEL
Department of Chemistry
Texas A&M University
College Station, TX 77843-3255, **USA**
Tel: (1-409) 8453373
Fax: (1-409) 8459452
E-mail: raushel@tamu.edu

Lily RAVEH
Department of Pharmacology
Israel Institute for Biological Research
74100 Ness Ziona, P.O. Box 19, **Israel**
Tel: (972-8) 9381747
Fax: (972-8) 9381559
E-mail: raveh@inter.net.il

Elsa REINER
Institute for Medical Research and
Occupational health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398

Pierre-Yves RENARD
SMM/DBCM/DSV, Bât 547 ext.
CEA Saclay
91191 GIF s/Yvette cedex, **France**
Tel: (33-1) 69082498
Fax: (33-1) 69085255
E-mail: renard@dsvidf.cea.fr

Paul G. RICHARDS
MRC Toxicology Unit
Hodgkin Building
University of Leicester
Lancaster Road, P.O. Box 138
Leicester LEI 9HN, **United Kingdom**
Tel: (44-116) 252 5569
Fax: (44-116) 2525616
E-mail: pgr3@le.ac.uk

Terrone L. ROSENBERRY
Research Department
Mayo Clinic Jacksonville
4500 San Pablo Road
Jacksonville, FL 32224, **USA**
Tel: (1-904) 9537375
Fax: (1-904) 9537370
E-mail: Rosenberry@mayo.edu

Sławomir RUMP
Department of Pharmacology and Toxicology
Military Institute of Hygiene and
Epidemiology
Kozielska 4
01-163 Warsawa, **Poland**
Tel: (48-22) 6108459
Fax: (48-22) 104391

Puttur SANTHOSHKUMAR
Department of Food Protectants and
Infestation Control
Central Food Technological Research Institute
570 013 Mysore, **India**
Tel: (91-821) 513210
Fax: (91-821) 517233
E-mail: skumar@nicfos.ernet.in

Tetsuo SATOH
Biomedical Research Institute
2802-1, Hiratsuka, Shiroi, Inba
Chiba 270-14, **Japan**
Tel: (81-474) 971089
Fax: (81-474) 971091
E-mail: satohbri@plu.ifnet.or.jp

Stefan SCHNEIDER
Leiter Büro Bonn
GTI Industrievertretungen
53229 Bonn, **Germany**
Tel: (49-228) 9481357
Fax: (49-228) 9481359
E-mail: BERECH@compuserve.com

Yoffi SEGALL
Israel Institute for Biological Research
24 Reuven St., P.O. Box 19
Ness-Ziona 74100, **Israel**
Tel: (972-8) 9381549
Fax: (972-8) 9401094
E-mail: segall@iibr.gov.il

Leon SENČIČ
Pedagoška fakulteta
Koroška cesta 160
2000 Maribor, **Slovenia**
Tel: (386-62) 22939
Fax: (386-62) 28180

Kristina SEPČIĆ
Department of Biology
Biotechnical Faculty
University of Ljubljana
Večna pot 111
1000 Ljubljana, **Slovenija**
Tel: (386-61) 1233388
Fax: (386-61) 273390
E-mail: kristina.sepcic@uni-lj.si

Avigdor SHAFFERMAN
Israel Institute for Biological Research
24 Reuven St., P.O. Box 19
74100 Ness-Ziona, **Israel**
Tel: (972-8) 9381595
Fax: (972-8) 9401094, 9401404
E-mail: avigdor@iibr.gov.il

Yechiel SHALITIN
Department of Biology
Technion - Israel Institute of Technology
32000 Haifa, **Israel**
Tel: (972-4) 8293701
Fax: (972-4) 8225153

Larisa V. SIGOLAEVA
Department of Chemical Enzymology
Faculty of Chemistry
M.V. Lomonosov Moscow State University
119899 Moscow, **Russia**
Tel: (7-095) 1132240
Fax: (7-095) 1132633
E-mail: sesns1rcmd@glasnet.ru

Israel SILMAN
Department of Neurobiology
The Weizmann Institute of Science
76100 Rehovot, **Israel**
Tel: (972-8) 343649
Fax: (972-8) 344161

Vera SIMEON-RUDOLF
Institute for Medical Research and
Occupational Health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398
E-mail: vesimeon@mimi.imi.hr

Palarp SINHASENI
Institute of Health Research
Chulalongkorn University
SOI Chulalonkorn 62
Phyathai Rd., Patumwan
Bangkok 10330, **Thailand**
Tel: (66-2) 2188152-3 or 2188147
Fax: (66-2) 2532395
E-mail: fphapsh@chulkn.car.chula.ac.th

Janez SKETELJ
Institute of Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1001 Ljubljana, **Slovenia**
Tel: (386-61) 310841
Fax: (386-61) 302272
E-mail: sketelj@ibmi.mf.uni-lj.si

Robert C. SORENSON
University of Michigan, Anesthesiology
Room 7433 Medical Sciences 1 Buldg.
1150 West Medical Center Drive
Ann Arbor, MI 48109-0615, **USA**
Fax: (1-734) 7649332

Malathi SRIVATSAN
Deptartment of Physiology
University of Kentucky
MS 514 Medical Center
800 Rose Street
Lexington, KY 40536-0084, **USA**
Tel: (1-606) 3235447 or 3235254
Fax: (1-606) 3231070
E-mail: msriv1@pop.uky.edu

Jure STOJAN
Institute of Biochemistry
Faculty of Medicine
University of Ljubljana
Vrazov trg 2
1000 Ljubljana, **Slovenia**
Tel: (386-61) 312357
E-mail: stojan@ibmi.mf.uni-lj.si

Manji SUN
Institute of Pharmacology and Toxicology
Academy of Military Medical Science
Taiping Road 27
Beijing 100850, **China**
E-mail: sunji@nic.bmi.ac.cn

Joel L. SUSSMAN
Department of Structural Biology
Weizmann Institute of Science
76100 Rehovot, **Israel**
Tel: (972-8) 9342638
Fax: (972-8) 9344159
E-mail: joel@sgjs3.weizmann.ac.il

Tivadar SZEGLETES
Research Department
Mayo Clinic Jacksonville
4500 San Pablo Road
Jacksonville, Florida 32224, **USA**
Tel: (1-904) 9532000
Fax: (1-904) 9537370

Mira ŠKRINJARIĆ-ŠPOLJAR
Institute for Medical research and
Occupational Health
Ksaverska cesta 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398
E-mail: mspoljar@mimi.imi.hr

Branka ŠURINA
Institute of Clinical Chemistry
Clinical Hospital "MERKUR"
Zajčeva 19
10000 Zagreb, **Croatia**
Tel: (385-1) 2302988
Fax: (385-1) 230220

Husref TAHIROVIĆ
Klinika za dječije bolesti Tuzla
75000 Tuzla, **Bosna i Hercegovina**
Tel: (387-75) 222810
Fax: (387-75) 222810

Palmer TAYLOR
Pharmacology Department 0636
University of California San Diego
School of Medicine
9500 Gilman Drive
La Jolla, CA 92093-0636, **USA**
Tel: (1-619) 5341366
Fax: (1-619) 5348248
E-mail: priley@ucsd.edu

Srđanka TOMIĆ-PISAROVIĆ
Department of Chemistry
Faculty of Science and Mathematics
University of Zagreb
Strossmayerov trg 14
10000 Zagreb, **Croatia**
Tel: (385-1) 4819289
Fax: (385-1) 4819288

Valerii TONKOPII
Laboratory of Water Ecotoxicology
Institute for Lake Research
Russian Academy of Science
Sevastianov str. 9
196199 St. Petersburg, **Russia**
Fax: (7-812) 2987327
E-mail: tonkopii@lake.spb.su

Miriam TOOMES
Department of Pharmacology
University of Heidelberg at Mannheim
Maybach St. 14-16
68169 Mannheim, **Germany**
Fax: (49-621) 377601

Cibby E. VARKEY-ALTAMIRANO
Eppley Institute
University of Nebraska Medical Center
600 S. 42nd St., P.O. Box 986805
Omaha, NE 68198-6805, **USA**
Tel: (1-402) 559-6014
Fax: (1-402) 559-4651
E-mail: cevarkey@unmc.mail.edu

Želimira VASILIĆ
Institute for Medical Research and
Occupational Health
Ksaverska c. 2, P.O. Box 291
10001 Zagreb, **Croatia**
Tel: (385-1) 434188
Fax: (385-1) 420398
E-mail: zvasilic@mimi.imi.hr

Eugenio VILANOVA
Instituto de Neurociencias
Facultad de Medicina
Campus Universitario de San Juan
03050 Alicante, **Spain**
Tel: (34-6) 5903889
Fax: (34-6) 5903896
E-mail: evilanova@ua.es

Janez VODIŠKAR
Institute of Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1000 Ljubljana, **Slovenia**
Tel: (386-61) 310841
Fax: (386-61) 302272

Urška WEBER
Institute of Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1000 Ljubljana, **Slovenia**
Tel: (386-61) 310841
Fax: (386-61) 302272

James R. WILD
Department of Biochemistry and Biophysics
College of Agriculture and Life Sciences
Texas A&M University
College Station, TX 77843-2128, **USA**
Tel: (1-409) 8451012
Fax: (1-409) 8459274
E-mail: j-wild@tamu.edu

Wei Hua XIE
Eppley Institute
University of Nebraska Medical Center
600 S. 42nd St., P.O. Box 986805
Omaha, NE 68198 - 6805, **USA**
Tel: (1-402) 5596014
Fax: (1-402) 5594651

Katarina ZAJC-KREFT
Institute of Pathophysiology
Faculty of Medicine
Zaloška 4, P.O. Box 2211
1105 Ljubljana, **Slovenija**
Tel: (386-61) 310841
Fax: (386-61) 302272
E-mail: zajc@ibmi.mf.uni-lj.sl

Ronald ZECH
Institut für Biochemie und
Molekulare Zellbiologie
Humboldtallee 23
37073 Göttingen, **Germany**
Tel: (49-551) 395981
Fax: (49-551) 395960

Vid ZGONC
Institute of Pathophysiology
School of Medicine
University of Ljubljana
Zaloška cesta 4, P.O. Box 2211
1000 Ljubljana, **Slovenia**
Tel: (386-61) 310841
Fax: (386-61) 302272

SPONSORS

AnAs - Analytical Assistance, Zagreb

CROATIA AIRLINES

FRANK ANALAB d.o.o. Zagreb

HERBOS d.d. Sisak

F. HOFFMANN- LA ROCHE L.T.D.
Predstavništvo Zagreb

HOTELI NJIVICE d.d. Njivice

PHARMAZAG, Zagreb

LKB Vienna

PLIVA d.d.

TLOS, Zagreb

Your flight can begin with the address
www.ctn.tel.hr/ctn
with information on flight timetables,
FF Club membership, news,
special offers and all that you may
wish to know about Croatia Airlines

CROATIA AIRLINES

FRANK ANALAB d.o.o.

Trgovina, servis i projektiranje
10000 Zagreb, Trg Francuske Republike 7 - Hrvatska

Matični broj: 3544630 Žiro račun: 30102-601-50108

PRODAJNI PROGRAM

"UNICAM" VELIKA BRITANIJA (u grupaciji **"Thermo Electron"USA.**)
Proizvodni program: spektrometrija (UV/VIS, FTIR, AAS, MS, ICP), kromatografija (GC, GC-MS) kapilarna elektroforeza, CHNOS i TOC analizatori.

"MILESTONE" ITALIJA
Proizvodni program: mikrovalni sistemi za brzu pripravu različitih uzoraka (krutih i tekućih), za sve vrste analiza, mikrovalni sistemi za brzo spaljivanje uzoraka, brzo odredjivanje vlage i/ili hlapljivih komponenti, brze ekstrakcije, brze organske sinteze itd.

"OHAUS" SAD (iz sustava grupacije METTLER-TOLEDO)
Proizvodni program: sve vrste tehničkih i analitičkih vaga za laboratorije

"SG" NJEMAČKA
Proizvodni program: uredjaji za pripravu čiste vode (ionska izmjena, reverzna osmoza, ultrafiltracija, kapaciteta od 6 - 20.000 l/sat.

BioChem ImmunoSystems SA, SERONO Diagnostika, NJEMAČKA
Proizvodni program: hematološki brojači od 5 do 20 parametara.

"TSP-Thermo Separation Products" SAD
Proizvodni program: separacijske tehnike i obrada podataka.

"WALDNER" GmbH, NJEMAČKA
Proizvodni program: laboratorijski namještaj.

"NETZSCH NEVAMATIC GmbH", NJEMAČKA
Proizvodni program: mašine za pranje laboratorijskog posudja, pranje i sterilizaciju medicinskog pribora.

"ENVIRONICS Oy", FINSKA
Proizvodni program: detektori za bojne otrove i ostale plinove.

SERVIS: Za svu opremu osiguran servis i rezervni dijelovi

ŠKOLOVANJE: Za sve korisnike osigurano školovanje kod nas i u tvornicama u inozemstvu

USLUGE: Savjeti pri projektiranju i puštanju u rad laboratorija.

Tel: +385 (0)1 3778-664, (0)1 3778-766, (0)1 3778-767, Fax: +385 (0)1 3770-368

HERBOS d.d. SISAK, HRVATSKA

HERBICIDI:

AFALON
ALON 50 DISP
ALON FORTE
ALON SUPER
BLADEX 50-SC
BRAVO
BRAVO MC
BRAVO RADAZIN T (SE)
BRAVO RADAZIN T
BRAVO TERAZIN - T
CIATRAL ALA
CIATRAL SCZ (SE)
CIATRAL SCZ
CIATRAL ATC
CIDOKOR
CLAP
DEVRINOL 45 FL
DIKOCID
FLEX
FUSILADE SUPER
GALIUM
GRAMOXONE
GRANSTAR 75 DF
GRODYL
HARMONY 75 DF
INAKOR-T
KAMATA
KEMIFAM
LENTAGRAN-WP
LONTREL 300
LONTREL 418 C
METOLAKLOR
MIKADO
OPTICA
PROHELAN-T
PUMA SUPER
RACER 25-EC
RADAZIN EXTRA
RADAZIN T-50
RADAZIN WP-50
RADAZIN WP-80
RADOKOR 50 WP
RADOKOR T-50
REGLONE FORTE
RODEO
SAFARI
SURPASS 6,7-E
TARGA SUPER
TERAZIN SC-50
TERAZIN WP-50
TERAZIN WP-80
TREFLAN EC

FOLIJARNA GNOJIVA:

BOROGREEN
CALCIOGREEN
FOLIACON
GREENPLANT
HASCON M 10 AD
MAGNEZIOGREEN

TAROT 25 DF
TROPHY
VENZAR

FUNGICIDI :

ANVIL 5 SC
BAKRENI DITHANE
BAKRENO VAPNO WP 50
BORDOŠKA SUSPENZIJA 20 WP
CAPTAN WP-50
CAPTAN 4-F
CHAMPION
CLARINET
CUPROLINE
DITHANE M-45
DITHANE M-45 FLO
GALBEN-C
GALBEN F
GALBEN-M
IMPACT
IMPACT-C
IMPACT 25
METEOR
MODRA GALICA
MYTHOS
NORDOX SUPER 75
PUNCH 10 EW
QUADRIS
RADOCINEB
RUBIGAN EC
SUMPOR SC-80
SUMPOR PRAH
SABITHANE
TRICHODEX WP

FUNGICIDI ZA TRETIRANJE SJEMENA:

CAPTAN SP-4
DITHANE S-80
MANKOZEB-TB
THIRAM 42-S
VITAVAX-200
VITAVAX 200-FF
VINCIT F

INSEKTICIDI :

ACTELLIC 50 EC
BATURAD-WP
FORCE 5 EC
HERBOREL - D
HOSTAQUICK EC-50
KARATE 2,5 EC
KARATE GOLD
KARATE MAX
LANNATE 20 L
MARSHAL 25 EC
MIMIC
OFUNACK 40-EC
ZLATICA OFUNAK-P
PIRIFOS EC
PIRIMOR 25 WG
PROTECT IT
RADOTION E-50
RADOTION P-5
RELDAN 40 EC
ROGOR 40
TALSTAR 10 EC

INSEKTICIDI ZA TRETIRANJE SJEMENA:

FORCE 20 SC
SEMAFOR 20 ST

SREDSTVA ZA ZIMSKO PRSKANJE:

BIJELO ULJE
CRVENO ULJE
PLAVO ULJE

AKARICIDI:

DEMITAN
OMITE 570EW

NEMATOCIDI:

METILBROMID-R

RODENTICIDI:

KLERAT PELETE
KLERAT WAX BLOCKS-20
KLERAT WAX BLOCKS-5
RATOX MAMAC-S
KLORFACINON ULJNI KONCENTRAT

FUMIGANTI:

DEGESCH PLOČE (trake)
DETIA GAS-EX P (pelete)
DETIA GAS-EX T (tablete)
DETIA MAGPHOS (pelete)
METILBROMID

REGULATORI RASTA:

NEO STOP
ROYALTAC
ROYAL MH 30
FAZOR

SREDSTVA ZA ZAŠTITU DRVETA:

DELTACID

INSEKTICIDI ZA PRIMJENU U KOMUNALNOJ HIGIJENI:

ICON 10 WP
ICON 0,6 ULV
K-OTHRINE WP-2,5
TRI-3
TRIFOXON COMPRESSE

SREDSTVA ZA POBOLJŠANJE VLAŽENJA I PRIANJANJA:

RADOVIT-N
TRIEND 90

OSTALA SREDSTVA:

ALL TRAP
ROACH TRAP (ljepilo za žohare)
TOPIN COLLA (ljepilo za glodavce i insekte)
PRSKALICA CP-3

HERBOS d.d. SISAK, HRVATSKA

ZA SVE DETALJNE INFORMACIJE OBRATITE SE HERBOSOVIM TERENSKIM AGRONOMIMA ILI DIREKTNO NA TVORNICU

"HERBOS" d.d. Sisak, Obrtnička 17,
telefon: 044/543-333, fax: 044/540-069

"HOTELI NJIVICE" d.d. NJIVICE • OTOK KRK – HRVATSKA

51512 NJIVICE, Tel: 385-51/846-112, 846-146, Fax: 385-51/846-116, 846-411

INFORMATION:

NjiviceTourist Community	**tel./fax**	**051/ 846-243**
"Njivice Hotel" Enterprise, Sales	**tel.**	**051/ 846-720, 846-112**
	fax.	**051/ 846-116**
Hotel "Jadran"	**tel.**	**051/ 846-105, 846-118**
Hotel "Beli Kamik"	**tel.**	**051/ 846-222, 846-202**
"Njivice" Autocamp	**tel.**	**051/ 846-168, 846-185**
"Plava terasa" Restaurant	**tel.**	**051/ 846-158**

Middle Europe's largest island on the Adriatic sea, the island of Krk is situated on Croatia's northern coast. Excellently connected in all directions its location is perfect for tourism. The island may be reached by plane, by land via the impressive Krk bridge and by ferry connections with neighboring islands. Some 20 km away Rijeka provides a very convenient rail connection. Although for centuries under various influences, the islanders have treasured their well known hospitality, customs and folklore. There is not a visitor who will not find the cultural and historical monuments most interesting, especially the Bašćanska tablet, written in the old Croatian Glagolitic alphabet, from the eleventh century.

Once a small fishing village with olivegroves and figgardens, Njivice is today an outstanding tourist resort. Your stay in Njivice will be well cared for by the local hotels and numerous small restaurants with their offer of fish and home specialities and the local wine Vrbnička žlahtina. Entertainment is varied, you can choose from terraces with music, traditional fisherman night fêtes, discos and night bars.

Njivice offers lots of sport activities: surfing, sailing, rowing, tennis, mini golf, bowling. And lovers of nature can enrich their stay with pleasant and healthy walks through oak forests along the shore.

Njivice eagerly awaits you.

CellCept© 250 mg

(mycophenolate mofetil capsules)

Roche

Roche Laboratories

A Member of the Roche Group

Please see complete product information available at this presentation.

varian

- GAS CHROMATOGRAPHS
- LIQUID CHROMATOGRAPHS
- GAS CHROMATOGRAPH / MASS SPECTROMETERS
- ATOMIC ABSORPTION SPECTROMETERS
- INDUCTIVELY COUPLED PLASMA SPECTROMETERS AND ICP-MS
- NUCLEAR MAGNETIC RESONANCE SPECTROMETERS
- UV – VIS – NIR SPECTROPHOTOMETERS
- SAMPLE PREPARATION PRODUCTS
- DATA SYSTEMS AND LIMS

BOMEM

- FTIR & FTNIR SPECTROMETERS
- RADIOMETERS
- ANALYZERS

PL

Polymer Laboratories

- LIQUID CHROMATOGRAPHS FOR GPC/SEC
- COLUMNS AND STANDARDS FOR GPC/SEC
- DATA HANDLING AND SYSTEMS FOR GPC/SEC
- DETECTORS FOR MOLECULAR WEIGHT DETERMINATION
- COLUMNS FOR HPLC

CEM INNOVATORS IN MICROWAVE TECHNOLOGY

- MICROWAVE DIGESTION SYSTEMS
- MICROWAVE ASHING SYSTEMS
- MICROWAVE EXTRACTION SYSTEMS
- MICROWAVE MOISTURE AND SOLIDS ANALYZER
- RAPID FAT ANALYSIS SYSTEM

Representative

AnAs
ANALYTICAL ASSISTANCE

Skokov prilaz 6
HR-10010 ZAGREB
Tel./fax +385-(01)-66-00-785
Tel. +385-(098)-27-88-51